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THE EFFECT OF NUTRITIONAL PROGRAMMING ON GUT MICROBIOTA IN BROODSTOCK AND PROGENY FISH

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by

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MAJOR PROFESSOR: Dr. Karolina Kwasek

Aquaculture is currently the fastest growing animal production sector. Because the aquaculture sector is growing at rapid rates, certain materials for feed, specifically marine protein sources, are becoming increasingly expensive and unsustainable. To counteract the reliance on fishmeal (FM) and other marine protein sources in the industry plant protein (PP), specifically soybean meal (SBM), has been investigated to replace FM as a protein source. Unfortunately, SBM when given in high quantities (greater than 30%) has been shown to negatively affect fish performance including retarded growth, intestinal inflammation, reduction of spawn quality, as well as dysbiosis in the gut microbiome, most likely due to presence of antinutritional factors such as saponins and tannins in SBM. The goal of this thesis was to investigate the effect of nutritional programming (NP) with SBM-based diet on gut microbiota in broodstock and progeny fish. Three feedings trials were conducted to test the efficacy of 3 approaches towards improving the use of PP in fish.

The first trial (Chapter 2), tested the effect of NP on larval zebrafish (*Danio rerio)*. NP is the theory of introducing an early nutritional stimulus to an animal that will 'program' the animal to better utilize the stimuli later in its adult life. The zebrafish were programmed in their larval stages, and the trial lasted for 65 days. There was a significant effect on growth performance for the programmed group (NP-PP) in terms of weight gains, as the NP-PP group grew better compared to the non-programmed group (NP-FM) and negative control (-control). There was no

significant effect on the gut microbiome in terms of alpha or beta diversity, however, there were significant changes in the relative abundance (RA) of the gut microbiome throughout time in the NP-PP and the NP-FM groups. The findings of the study support that early NP of zebrafish with SBM improves growth performance on PP diet, but the gut microbiome does not seem to be a mechanism for NP.

The second feeding trial (Chapter 3) focused on NP induced in the zebrafish broodstock with dietary SBM. For two weeks, the broodstock fish were fed with either a SBM diet or a FM diet so that gametogenesis occurred with either a FM or PP diet. This phase was called the 'broodstock programming' stage. The broodstock were then spawned, and the larval fish were separated into four different treatments: 1) SBM broodstock progeny, fed SBM for the entire trial (PPBS-PP) 2) SBM broodstock progeny fed FM the entire trial (PPBS-FM), 3) FM broodstock progeny fed FM the entire trial (+ control, FMBS-FM), and 4) FM broodstock progeny fed SBM the entire trial (- control, FMBS-PP). The PPBS-PP group achieved similar weight gains compared to all other treatments in terms of grams, but was numerically greater than the FMBS-PP treatment. There were no differences detected in gut microbiome alpha or beta diversity in any of the groups, however, there was significant change observed of certain bacterial phyla between the 'programmed broodstock', larval fish, and fish at the end of the trial, 48 days post hatch. Overall, this trial suggests that parental programming does not improve PP utilization in the progeny of zebrafish. It also appears that the gut microbiome is not a mechanism of parental programming.

The third feeding trial (Chapter 4), was conducted on largemouth bass (*Micropterus salmoides).* This chapter had a similar experimental design as the first trial (Chapter 2), and larval largemouth bass were programmed with dietary SBM. This trial had an additional group

added to it, which included a dietary saponin-programmed group. The study found that the NP with SBM diet or dietary saponin did not improve PP utilization and growth performance of largemouth bass in its pre-adult age. The study also found that the NP with SBM diet or dietary saponin did not have any effect on the largemouth bass gut microbiome, and there does not seem to be any gut microbiome modification associated with the NP in this fish species.

Overall, NP can be used to improve dietary PP utilization but optimal timing and PP delivery method must be well assessed to ensure successful PP exposure and adaptation in different species. Nevertheless, the gut microbiome does not seem to be affected by NP and therefore is not considered the mechanism behind NP. Finally, studies on both zebrafish and largemouth bass presented major shifts in the gut microbiome as the fish aged. In addition, the core microbiomes of both species appeared to become more pronounced as the fish become adults. There seem to be an evolutionary tie between host and its gut microbiome. More studies, however, should further investigate this and the genetic effects on gut microbiota development and its heritability.

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CHAPTER 1

INTRODUCTION

Aquaculture is the breeding, rearing, and harvesting of fish, shellfish or other aquatic organisms in some form of water environment (NOAA 2018). Aquaculture, like other forms of farming, has been around for centuries and is thought to have started around 1500 BCE in ancient China where carps were farmed (NOAA 2018). The form of farming that spurred aquaculture was mostly extensive pond farming. Extensive farming is usually in static water in which fish are fed through natural processes (naturally occurring zooplankton and phytoplankton) and therefore does not require external food or protein/energy inputs. Because of no food input, however, extensive ponds are not characterized by high yield of fish. Consequently, the industry has been moving towards more intensive fish production that requires a more substantial input of other resources including formulated feeds. Historically, fish feeds have been formulated using marine raw materials such as fishmeal and fish oil obtained from processing of lower value marine fish species captured from the wild. Subsequently, due to rapid growth of the aquaculture industry the need for fishmeal has been continuously growing leading to diminished natural resources and drastic increases in prices of this marine raw material (Burnell and Allen 2009).

Aquaculture had a major expansion in production from 1980-1989 (11.2% annually) and from 1990-1999 (10% annually) (FAO 2018). Although the rate of growth for aquaculture has slowed down in the last few years, to approximately 4-5% annually, this growth rate still exceeds the development rate of all other animal production systems. In the 1990s, aquaculture produced 100 million tons of fish, in 2018 production increased to about 175 million tons, and the projected amount of fish to be produced in 2030 is 200 million tons (FAO 2018). This more

intensive fish production and the constant growth of the industry increase the need for more innovative protein sources for aquaculture feeds including plant proteins (PP) that would successfully replace marine protein ingredients including fishmeal.

One of the challenges of using fishmeal in the feeds is the general availability of the fish being used to make the meal. The total amount of whole fish (small, schooling, marine pelagic fish) has decreased by 50 percent from 1996-2018 (FAO 2018). Whole fishes are not being used because seasonality affects their abundance. This is also paired with the hard overfishing which causes low amounts of fish being caught for fishmeal purposes. This has been partially combated by processing of fish by-products as a source of fishmeal. This is a short-term solution, however, due to low by-product generation to offset the depletion of wild fishmeal producing fish.

Fishmeal comes from several sources but is usually derived from small pelagic fishes such as anchovetas (*Engraulis ringens)*, menhaden (*Brevoortia spp.)*, anchovies (*Engraulis spp.)* and sardines (*Sardina spp.)* (FAO 2018). Due to the growing aquaculture industry and its high demand for fishmeal, these fish populations are under immense pressure, which grows as the industry keeps expanding. Furthermore, fishmeal is significantly more expensive compared to some PP sources including soybean meal (SBM) and others. Fishmeal and SBM back in the 1950s until the early 2000s were almost mirrored in price. Fishmeal was approximately 100-200 dollars more per ton compared to soy (Asche, Oglend and Tveteras 2012). The price of fishmeal is now almost four times the amount of SBM (Asche, Oglend and Tveteras 2012).

Over the past decades, aquaculture has focused on replacing fishmeal with alternative protein sources derived mostly from plants due to their lower cost and higher availability. High-quality PP concentrates such as soy protein concentrate or wheat gluten are already being widely used by the feed industry since their digestibility in some species is comparable to fishmeal.

Although some studies show positive results on fish growth with lower quality dietary PP sources, including SBM, these raw materials at higher inclusion rates often impair proper development and growth potential of the fish (Geurden et al. 2013, Kasper et al. 2007). PP, specifically SBM, can carry antinutritional factors, such as saponins (Perrera and Yufera 2016). These antinutritional factors can be a source of intestinal inflammation, depression of feed efficiency and elimination of beneficial gut microbes. (Merrifield et al. 2017, Geurden et al. 2013).

Many different aspects influence the gut microbiome of fishes and environment, feeding habits, and genetics shape the majority of it (Gallo et al. 2019). The age of fish also has a drastic effect on the development of the fish gut microbiome structure. For example, Stephens et al. (2016) revealed that as zebrafish age, their gut microbiome composition and abundance is significantly affected, specifically with the morphological changes of the intestine. Similarly, Zarkasi et al. (2016) reported that the age of the fish and even seasonality and sampling time all have a strong determination of the gut microbiome composition.

Other factors including species, epigenetics, parental habits, and breeding areas are also contributors to the gut microbiome profile (Smith et al. 2017, Merriefield et al 2011). Furthermore, many studies have also shown that PP, specifically SBM, induces negative effects on the fish by decreasing numbers of different aerobic and anaerobic bacteria strains in the intestine (Merrifield et al 2011). Piazzon et al. (2017) revealed that 58% soybean meal-based diet provided to gilthead seabream (*Sparus aurata)* corresponded with a higher parasite and infection rate and lower gut microbiota diversity compared to fish that were fed a fishmeal diet, indicating the harmful influence that vegetable meals might have on the gut microbial community, and subsequently, the fishes ability to respond to diseases. Generally, a positive correlation has also

been reported in higher vertebrates between diversity of the gut microbiome and animal health, and longevity (Ghanbari, Kneifel, and Doming 2015).

As the human population grows, fish as a protein and fatty acid source are becoming more important, specifically for impoverished nations. Because of the expense and lack of marine protein sources, the industry ought to consider drastic changes towards a more sustainable and cost-effective protein source, such as PP for fish feeds. Therefore, the goal of this thesis was to determine methods in which the negative effects of lower-quality PP could be mitigated. Three trials were run in this thesis. Chapter 2 tested the effect of NP in larval zebrafish (*Danio rerio)* with dietary SBM, and its effects on the gut microbiome and growth performance of the fish in pre-adult stages on PP diet. Chapter 3 tested the effect of parental NP on zebrafish progeny gut microbiome and fish growth on PP diet. Chapter 4 used a similar approach as chapter 2, but larval largemouth bass (*Micopterus salmoides)* were used as a model fish species and the NP effect of dietary SBM and soy saponin were used to test for the effects of gut microbiome and growth. The introduction in each chapter provide a more detailed explanation of each approach taken.

CHAPTER 2

NUTRITIONAL PROGRAMMING WITH DIETARY SOYBEAN MEAL AND ITS EFFECT ON GUT MICROBIOTA IN ZEBRAFISH (*DANIO RERIO)*

ABSTRACT

Nutritional programming (NP) is considered as a promising approach that can counteract the negative effects of dietary plant protein (PP) by introducing PP to fish in the early developmental stages and enhancing PP utilization later in life. However, the mechanism of NP is still unclear. The objective of our study was to assess the effect of NP on PP utilization and the gut microbiome in zebrafish *Danio rerio*. At 4 days post hatch (dph) zebrafish larvae were randomly distributed into 12 (3 L) tanks, 157±16 larvae per tank. The study included four treatment groups: 1) A positive control group that received a fishmeal diet (FM) throughout the entire trial (+ control); 2) A negative control group that received PP diet throughout the entire trial (-control); 3) A NP group that received dietary PP during the larval stage followed by FM-based diet during the juvenile stage and PP diet again during a PP challenge in the grow-out phase (NP-PP); and 4) A FM-group that received FM-based diet and was challenged with a PP diet during the grow-out phase (NP-FM). During the PP challenge the NP-PP group achieved the highest weight gain compared to the (-) control and NP-FM groups. The relative abundance (RA) of fish gut microbiomes overall did not present any statistical differences throughout the experiment, but the RA of certain phyla such as Chloroflexi, Planctomycetes, and Bacteroidetes presented higher values in some groups at early juvenile stage. The fish gut microbiome also presented differences throughout the fish's life no matter what feed was provided, presenting a natural progression of the zebrafish gut microbiome with age.

INTRODUCTION

Fishmeal, main protein source commonly used in fish diets, have been continuously replaced with alternative protein sources derived from plants due to their lower cost, higher availability and sustainability. Although some studies show positive results on fish growth with lower quality dietary plant protein (PP) sources, including soybean meal (SBM), these raw materials at high inclusion rates negatively affect proper development and growth potential of many fish species (Geurden et al. 2013). For example, SBM can eliminate some beneficial gut microorganisms, leading to detrimental bacteria taking control of the gut, which can lead to infections as well as intestinal inflammation (Merrifield et al. 2011). High levels of terrestrial PP have also been shown to depress feed efficiency and provoke morphological changes in the distal intestine of the gut (Geurden et al. 2013). Stress and disease resistance are also lowered when high levels of PP are incorporated in the diet and can also reduce the integrity of the fillet in terms of omega-3 fatty acids (Turkmen et al. 2017). Some PP sources, such as SBM, also carry anti-nutritional factors such as saponins, tannins, trypsin inhibitors, etc. which decrease the ability of the intestinal brush border cells to absorb nutrients (Perera and Yúfera 2016).

Nutritional Programming (NP) is described as early feeding events during larval or juvenile stages that may result in altered physiological responses including growth, intestinal health, metabolism and other effects in the adult fish (Kemski et al. 2016). NP has been studied on mammals extensively, but early programming in fish is now more researched with several research topics in fish stemming from mammalian studies (Hou and Fuiman 2020). NP with PP shows great promise to increase utilization of lower-quality PP sources (Kemski et al. 2018; Geurden et al. 2013; Kwasek et al. 2020; Molinari et al. 2020; Izquierdo et al 2015). Balasubramanian et al. (2016) challenged rainbow trout (*Oncorhynchus mykiss*) with vegetable

oil-based diet and reported that the fish later presented higher feed intake, growth, and feed utilization compared to non-programmed individuals. Interestingly, Geurden et al. (2014) provided insight that early programming of rainbow trout alevins lead to similar alpha diversity measures of gut microbes between programmed versus non-programmed individuals, showing that programming may positively affect the gut microbiome when exposed to plant protein. We also now know that NP positively affects utilization of PP-based diets in a model species zebrafish *Danio rerio* (Kwasek et al. 2020) but the mechanism behind NP remains unclear.

The gut microbiome develops in fish during the egg stage and later larval stage where it is colonized by bacteria that are in the surrounding water (Ghanbari et al 2015). As the fish matures and the gut advances, more bacteria colonize the gut, originating from the surrounding water, feed, and other fish. There is also a strong correlation between the gut physiology and the gut microbiome profile it forms (Leulier et al. 2017). Several studies have shown that gut microbiome manipulation can have drastic effects on fish performance (Xia et al. 2014, Perez et al. 2010, Gomez et al. 2008) . Several factors influence the gut microbiome in fish including species, age, genetics, epigenetics, parental habits, breeding areas, habitat, and diet (Smith et al. 2017, Merrifield et al. 2011). Several studies have also shown that PP, specifically SBM, induces negative effects on the fish by decreasing numbers of different aerobic and anaerobic bacteria strains in the intestine (Merrifield et al. 2011). More specifically, a decrease in gut microbial diversity has been shown to increase fish vulnerability to disease, impaired nutrient processing, and in severe cases, death. A positive correlation has also been reported in higher vertebrates between diversity of the gut microbiome and animal health, and longevity (Ghanabri et al. 2015). Although there are several reports available on the fish microbiome, the connection between NP and the gut microbiome has not been evaluated.

Our hypothesis is that the improved performance induced by NP is associated with modification of the gut microbiota in response to specific nutritional trigger (in this case PP) in the larval stages that lead to development of "core" gut microbiota that remains throughout the fish development leading to an enhanced growth of fish in their pre-adult stages when the same nutritional trigger (PP diet) is re-introduced. The objectives of this study were two-fold: 1) To determine the impact of NP with dietary PP induced during the larval development on the growth and gut microbiota of zebrafish *Danio rerio*; and 2) To assess if the gut microbiome changes in fish throughout its development upon receiving different dietary treatments. Zebrafish were chosen for this project due to their wide use as a model species in biomedical, genetic, and nutritional studies including those on NP (Kwasek et al. 2020). They are characterized by fast generational time, high fecundity, and relatively easy maintenance (Hedrera et al. 2013).

MATERIAL AND METHODS

The study was conducted in the Center for Fisheries, Aquaculture, and Aquatic Sciences at Southern Illinois University - Carbondale (SIUC) in a recirculated aquaculture system (RAS; Pentair Aquatic Eco-systems, Cary, NC). The RAS system was fully automated for pH and conductivity and was equipped with two mechanical filters, a carbon filter and UV light. The water inflow was adjustable and all flow rates were set the same in each tank. All experiments were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of SIUC. The SIUC Institutional Animal Care and Use approved all of the protocols (Protocol # 18-007) performed. During fish handling, anesthesia was performed using water bath immersion in tricaine methane sulfonate (MS222) at a recommended concentration (0.01 mg/ml), and all efforts were made to minimize pain, stress, and discomfort in the animals.

The average water temperature during the experimental period was 27.1 ± 0.2 ºC. The average pH and conductivity were 6.99 ± 0.34 and 1,602 ± 322.18 S/m, respectively. The salinity was kept at 2-3 parts per thousand (ppt) throughout the live feeding to prolong viability of live food and at 1-2 ppt during the rest of the experiment. The photoperiod consisted of 14 hours of darkness and 10 hours of light, with overhead lights on from 8:00-18:00.

Broodstock Rearing and Spawning

Larval fish were obtained from broodstock at SIUC. Broodstock fish were kept in separate tanks and fed 2-3 times a day two weeks before breeding. When the fish spawned a 2:1 ratio of females to males, 17 females and 8 males were combined as suggested by (Westerfield 2000). A wire net with a 1.5-millimeter mesh was placed in the breeding tank (10 Liters), with a fake plant to induce spawning. The fish were left to breed for 24 hours and then the broodstock were removed. The wire mesh was taken out, and eggs hatched after being laid at 27ºC. At 4 days post hatch (dph), when the majority of the larvae were actively swimming, they were randomly distributed into experimental tanks (3 Liters) with 157±16 larvae per tank. A total of 12 tanks were used, each treatment (4 in total) had triplicates.

Larval zebrafish were fed to apparent satiation with saltwater rotifers (*Brachionus plicatilis)* starting from 4-7 dph. The rotifers were obtained from cysts purchased from a commercial vendor (Brine shrimp direct, Ogden, Utah). The rotifers were kept in a 30 Liter bucket. The salinity was held at 15 parts per thousand (ppt), the temperature was kept at 23-25ºC, and constant aeration and light were supplied to the bucket. The rotifers were fed twice a day with powdered Spirulina sp. (Earthrise Nutritionals, Irvine, California). The water was changed daily. Zebrafish were then fed to apparent satiation with *Artemia* nauplii together with rotifers from 7-10 dph and then just *Artemia* from 10-12 dph. To induce *Artemia* hatching,

Artemia cysts (GSL brine shrimp, Ogden, Utah) were added to Macdonald jars and incubated for 24 hours under constant light at 25^{o}C and salinity of 30 ppt. After 24 hours, the *Artemia* hatched into nauplii and were harvested through a 150-micrometer sieve, washed under freshwater, and scooped into the tanks for feeding.

Diet preparation

All the experimental feeds were formulated according to Kwasek et al. (2020) and produced at SIUC. Two different types of diets were made: a diet formulated to contain SBM as a main protein source (PP diet, SBM diet), and a diet based on fishmeal as a main protein source (FM diet; Table 2.1)

All dry protein ingredients (fishmeal, krill meal, and soybean meal) were added to a centrifugal mill, (Zm 100, Retsch Haan, Germany) and ground to 0.5 micrometers. After the centrifugal mill, all ingredients were manually sieved through a .255-micrometer sieve to ensure all particles are uniform and of the appropriate size.

All the dry ingredients (excluding soy lecithin and choline chloride) were added together and mixed for 15 minutes. After all the dry ingredients were mixed the fish oil was added with the soy lecithin dissolved in the oil to ensure even amounts of lecithin throughout the feed. The oil and dry ingredients were mixed again for 15 minutes. After the oil and dry ingredients were done mixing, water with dissolved choline chloride (15% of total mass of feed) was added ensure even mixing. Next, the feeds were processed using an extruder (Caleva Extruder 20, Sturminster Newton Dorset, England) to produce “noodles”. Feed was slowly added to the extruder at levels between 20-24 RPM to obtain a proper noodle size. After the noodles were made, they were processed using a spheronizer (Caleva, Sturminster Newton Dorset, England) at 600 RPMs for 3 min, 1800 RPMs for 30 seconds, and then 600 RPMs for 2-5 minutes to finish the process. The

noodles were added to the spheronizer to make proper size of uniform spheres for feeding, and to encapsulate the feed to avoid nutrient leeching in water. Finally, the pellets were dried using a freeze dryer (Labconco, Kansas City, MO).

After drying pellets were sieved to appropriate size using a vibratory sieve shaker (Retsch Hann, Germany). The shaker assorted the pellets ending at a powder form (<.155 Micrometers) and starting at the biggest pellet size (>.80 micrometers) with several sizes in between. All finished feeds were stored in bags in -20° C to avoid oxidation. While the feeds were used in experimentation, they were kept at 4 ºC to keep the integrity of the pellet.

Proximate composition of diets included quantification of the following: crude protein, crude lipid, moisture, and ash. Briefly, samples were analyzed for ash by combustion (550 °C for 5 h) in a muffle furnace (Lindberg Blue M, MA); crude protein (N×6.25) using a Leco nitrogen analyser (Model FP-628, Leco Corporation, St. Joseph, MO); and crude lipid was extracted with chloroform–methanol (2:1, v/v). All dietary samples were analyzed in triplicates.

Experimental Groups and Feeding Regime

The trial consisted of four different treatments as follows:

1) A positive control group that received a fishmeal diet throughout the entire trial (positive control; + control); 2) A negative control group that received PP diet throughout the entire trial (negative control; -control); 3) A NP group that received dietary PP during the larval development followed by fishmeal-based diet during the juvenile stage and PP diet again during a "PP challenge" in the grow-out phase (NP-PP); 4) A FM group that received fishmeal-based diet during the larval and juvenile stages and was challenged with a PP diet during the grow-out phase (NP-FM) (Figure 2.1). There were three replicates for each group, each tank considered as an individual observation unit (3 total) counted as a replicate for a treatment.

Fish were fed up to satiation until 22 dph. From 22-65 dph, all fish were fed at a restricted rate (8-9%) The feeding rate was originally set by measuring the observed feed intake for each tank and setting the feeding level to the tank with the lowest feed intake. This ensured a consistent feeding rate across all tanks and consumption of all the feed provided. In addition, the feeding rate was adjusted daily, using an assumed FCR of 1, and also readjusted through observations of feed intake at each feeding. Also, a bi-weekly weighing was conducted during the restricted feeding period, in order to determine the actual biomass in each tank and, readjust the feeding rate accordingly.

Sampling and Measurements

The measured responses that were assessed included: final average weight, weight gain, survival, and differences in gut microbial diversity and community composition. Fish were measured on a weekly basis to adjust the feeding rates correctly. Average weight gain was calculated per treatment starting the day of the PP challenge till the end of the study. Average weight gain was calculated as follows:

Final mass (g)-initial mass (g) = Weight gain (g)

((Final mass- initial mass)/initial mass) *100= Weight gain (%)

The whole larval fish and whole intestinal tracts (pre-adults) were sampled throughout the study to assess the structure of the gut microbiome for each treatment (Figure 2.2). Three fish were netted randomly from each replicate tank and frozen directly in liquid nitrogen to preserve the gut microbiome (Rimoldi et al. 2018). After that, fish were kept at -80^{o}C freezer until the analyses.

DNA Extraction, PCR Amplification, 16S rRNA gene sequencing and amplicon analyses

Fish were minimally thawed from the freezer on dry ice before dissection. Adult fish were dissected, and the intestines were removed from the fish. The intestines were then lysed and the DNA was extracted using protocols of MO BIO Power soil isolation kit (Hilden, Germany). Larval fish went through the same procedure except they were washed in 70% ethanol for one minute and then washed for 30 seconds under distilled water to remove any attached bacteria as suggested by Ringo and Birkbeck (1999). DNA concentrations were quantified using a Qubit fluorometer (ThermoScientific, Waltham MA). The DNA was brought to the Shedd Aquarium's Molecular and Microbial Ecology Laboratory (Chicago. Illinois) for bacterial DNA analysis.

Bacterial and archaeal DNA was amplified using primer constructs (515f/806rB) targeting the V4 region of the 16S rRNA gene (Walters et al. 2016). The constructs contain Illumina specific adapters followed by 12bp Golay barcodes on each forward primer, primer pads and linkers as well as the template specific PCR primer at the 3' end. PCR was performed in replicate 25µl reactions containing 12.5µl Phusion Hot-Start Flex 2X MasterMix (New England Biolabs), 0.2µM final concentrations of forward primer 515f and reverse primer 806rB, 2µl of template DNA and nuclease free water to equal 25µl. Mock microbial community DNA standards (Zymo Resarch) and negative controls containing no template DNA were prepared with each PCR replicate. Thermal cycling conditions were carried out as follows: 98°C for 30 seconds, 30 cycles at 98°C for 10 sec, 55°C for 30 sec and 72°C for 30 sec, with a final extension of 5 minutes at 72°C. After PCR, replicate amplicons were combined and 5µl of each were electrophoresed in 1.8% agarose gels to confirm amplification of the V4 region. 20µl of each amplicon was then cleaned and normalized using the SequalPrep™ Normalization Plate Kit (Applied Biosystems), and equal volumes of each normalized amplicon were pooled together.

The pooled amplicon library was quantified using a Qubit™ 3.0 fluorometer and Qubit™ dsDNA HS Assay Kit (Life Technologies), then further cleaned and concentrated using the UltraClean® PCR Clean-Up Kit (MO BIO Laboratories). The molarity of the pooled library was calculated and was diluted to 2 nM before denaturation and further dilution to a loading concentration of 6 pM. Paired-end sequencing for a total of five hundred cycles was conducted on the Illumina MiSeq platform using custom sequencing primers described previously (Caporaso et al. 2012). with addition of 10% PhiX Control library (Illumina) to increase sequence diversity.

Raw sequence reads were processed using a combination of QIIME2 version 2018.11(Boylen et al. 2019) and phyloseq (McMurdie et al. 2013). Demultiplexed reads were imported into QIIME2 and denoised with DADA2 (Callahan et al. 2016) (via q2-dada2), and reads were trimmed at 13 bp and truncated after 230 bp. DADA2 filters sequences for quality, removes chimeric sequences, merges paired-end reads, and produces amplicon sequence variants (ASVs). A rooted phylogenetic tree was then generated using the 'q2-phylogeny' pipeline under default settings, and taxonomy was assigned to ASVs using a Naïve Bayes classifier trained on the SILVA release 132 99% OTUs database (Quast et al. 2012) where sequences had been trimmed to include only the bases within the V4 region bound by the 515F/806R primer pair. Reads that mapped to chloroplast and mitochondrial sequences were filtered from the sequence variants table using the 'filter_taxa' function. Data was then imported into phyloseq using the "import_biom" and 'import_qiime_sample_data' functions and merged into a phyloseq object. Samples with less than 3639 reads were discarded, and alpha diversity was calculated using the Shannon index and observed ASVs. The data was then proportionally transformed to a normalized read count of 3639. Beta-diversity was analysed using unweighted UniFrac distances

calculated in phyloseq. From these distances, principal coordinates ordinations were calculated and plotted. Taxonomic bar plots were generated using relative abundance from each of the remaining samples.

Statistical analysis

One-way ANOVAs were run for final average weight and weight gain data, followed by a Tukey's post-hoc test with a significance set at $p<0.05$ (SPSS, Chicago, IL version 25). R (Boston, MA) version 3.5.2 was used for statistical analysis of alpha and beta diversity. One-way ANOVAs were generated with the R package multcomp using a Tukey's post-hoc test with Westfall values for relative abundance (RA) and statistical analysis with a PERMANOVA was used for beta diversity measures.

RESULTS

Growth performance

The average fish weight for each treatment was assessed throughout the study on a weekly basis starting at 24 dph and ending at 65 dph (end of PP challenge). There were no statistical differences detected in average fish weight throughout the study (ANOVA, n=3, df=8, 11).

The average weight gain was assessed during the PP challenge measured from the final weigh day (end of PP challenge, 65 dph) to the first day of the PP challenge (36 dph). The NP-PP group had significantly higher weight gain (g and %) compared to both the NP-FM (grams $p=0.003$, % $p=0.002$) and (-) control groups (grams $p=0.001$, % $p= 0.006$) and not different compared to the (+) control group (grams $p=0.873$, % $p=0.905$) (Table 2.2). The weight gain (g and %) of (+) control group was significantly higher compared to the (-) control (grams $p=0.001$,

% p=0.003) but not different than NP-PP (grams p=0.28, % p=0.905) or NP-FM (grams p=0.021, % p=0.117) groups.

The positive control had a higher weight gain (g) compared to the (-) control (p=0.001) but no difference was found between (+) control, NP-FM (p=0.21), and NP-PP (p=0.28) groups. The (-) control had the lowest weight gain (g and %) among all groups (Table 2.2).

Alpha diversity of gut microbial communities

Shannon index values and the total number of observed ASVs were calculated to represent alpha diversity between the different treatment groups at each time point. Comparisons were carried within each diversity metric out at each time point using one-way ANOVAs. The data was split into four time points: 1) the pre-dry (or live food) diet time point (samples from 4 dph - no feed, 6 dph - rotifers only, 12 dph - rotifers and artemia; Figure 2.3); 2) The post-programmed time point (24 dph; Figure 2.4A); 3) the pre PP-challenge time point (36 dph; Figure 2.4B); and 4) the end of the study (65 dph; Figure 2.4C). There were no significant differences in Shannon values or observed ASVs between any of the four groups at any of the time points.

Beta diversity of gut microbial communities

PCoA plots were generated for the data as a whole and at each of the four time points. The PCoA including all the samples demonstrated that samples from fish on either no diet or live diet (4 dph and 6 dph, 12 dph) clustered together, and samples taken from the 24 dph, 36 dph and 65 dph, did not appear to cluster based on any discernable pattern

Statistical analysis with a PERMANOVA confirmed there were no significant differences in community composition structure between treatment groups at 24, 36, and 65 dph (PERMANOVA, p-value > 0.05, n= 5-11). At the pre-dry diet time point, however, there was a

significant difference in beta diversity between the diet types (4 dph, 6 dph, 12 dph PERMANOVA, p-value > 0.05, n= 8) (Figure 2.5).

Relative abundance of the gut microbiota

Throughout each time point relative abundance (RA) of gut microbiota was analyzed. The majority of the larval fish and larval fish gut RA at the 'pre-dry diet' stage consisted of Proteobacteria (18-67% at 4 dph, 55-59% at 6 dph, and 32-41% at 12 dph) and Bacteroidetes (20-77% at 4 dph, 18-41 % at 6 dph and 12-32% at 12 dph). Chloroflexi contributed up to 1-5% of RA at the 'pre-dry diet' stages and there was statistical difference in RA of Chloroflexi between fish at 4, 6 and 12 dph. Chloroflexi was highest in the 'rotifer and Artemia' fed group at 12 dph (34%) compared to 'no diet' at 4 dph (3%) or rotifer group at 6 dph (7%) (Figure 2.6). Other phyla such as Firmicutes, Planctomycetes, Thaumarchaeota, Actinobacteria, Verrucomicrobia, and Patescibacteria contributed to less then 1% of the RA at 4, 6, and 12 dph stages (Figure 2.7).

At 24 dph the RA of fish guts consisted mostly of Proteobacteria (32-56%), Fusobacteria (1-30%), Bacteroidetes (12-18%) Firmicutes (2-8%) Chloroflexi (1-19%). Planctomycetes had a RA of 0.09-1.4%. All other bacteria (Thaumarchaeota, Actinobacteria, Verrucomicrobia and Patescibacteria) made up <1% of the RA (Figure 2.8)

The only difference detected between treatment groups at 24 dph was the gut RA of Bacteroidetes and Planctomycetes. The NP-PP fish had the highest amount of Bacteroidetes (54%) compared to (+) control (15%) but not different compared to fish from NP-FM and (-) control (Figure 2.9A) while the (-) control group had the highest RA of Planctomycetes (2%) compared to all other groups (Figure 2.9B).

At 36 dph only NP-FM and NP-PP treatments were analyzed. The RA of both groups mostly consisted of Proteobacteria (20-34%) and Fusobacteria (45-71%). Bacteroidetes RA was present at 2-4%, Firmicutes at 4-6%, and Thaumarchaeota at 6%. All other bacteria (Chloroflexi Actinobacteria, Verrucomicrobia Patescibacteria and Placntosymetes) were present at <1% RA (Figure 2.10).

At 65 dph, all four-treatment groups were analyzed for gut microbiome RA. However, no significant differences were detected between the groups. The RA consisted mostly of Proteobacteria (35-95%), Fusobacteria (2-54%), Bacteroidetes (0.2-11%), Firmicutes (0.5-18%), Thaumarchaeota (0-2%) and Actinobacteria (0-12%). The remaining phyla, including Patescibacteria, Verrucomicrobia, Planctomycetes, and Chloroflexi were all present at <1% RA (Figure 2.11).

Relative abundance of the gut microbiome over time

The study analyzed NP-FM and NP-PP groups to evaluate gut microbiome development over time for the six most abundant phyla. In the NP-FM group, Proteobacteria was higher at 6 and lowest at 24 and 36 dph (ANOVA, p=.045, p=.037, n=5). All other time points had statistically similar Proteobacteria RA (ANOVA, p>0.05, n=5). Fusobacteria RA was the lowest at 6, 12 and 24 dph compared to 36 and 65 dph (ANOVA, p=0.188 at 12 dph, and p=0.980 at 65 dph, n=5). Bacteroidetes was higher at 6 and 24 dph but almost disappeared from the gut and was lower at 36 and 65 dph (ANOVA, p=0.015, n=5). No differences were found in Bacteroidetes RA between fish at 12 dph and the other ages (p=.708). Chlorloflexi was the highest at 12 dph, but the same at all other time points (ANOVA, p=0.004 n=5). Planctomycetes and Firmicutes RAs were similar throughout the trial (ANOVA, p=0.210, n=5) (Table 2.3).

In the NP-PP group Proteobacteria RA in fish gut was similar throughout the trial (ANOVA, p=0.188N=5). Fusobacteria RA was higher at 36 and 65 dph compared to the earlier stages (ANOVA, p=0.004, n=5). A pattern similar as in the NP-FM group. Bacteroidetes RA was higher during the early stages at 6 and 24 dph and dropped at 36 and 65 dph (ANOVA, p=.002 n=5). Cholorflexi RA was higher at 12 dph compared to all other stages (ANOVA, p=0.004, n=5). Firmicutes RA was higher in the later stages at 36 and 65 dph compared to 6, 12, and 24 dph. Finally, Planctomycetes RA was the highest at 65 dph compared to all the earlier stages (ANOVA, p=0.042, n=5) (Table 2.3).

DISCUSSION

Growth performance

The present study found that the NP-PP group achieved a higher weight gain compared to the NP-FM group and the (-) control while the (-) control presented the lowest weight gains compared to all other groups. These results confirm previous studies which showed that NP with dietary PP is able to "imprint" the zebrafish to improve dietary SBM utilization at a later stage (Kwasek et al. 2020) for better growth as opposed to continuous exposure to SBM diet ((-) control) which in most cases leads to poor growth performance. Multiple research has shown that some partial fishmeal replacement with SBM (at or below 25-30%) in diets does not necessarily induce negative effects on fish growth, however, higher SBM inclusion (above 30%) or complete fishmeal replacement might lead to significant growth retardation in some species (Kasper et al. 2007). At 100% PP diets, rainbow trout showed lower specific growth rates compared to fish that received diets based on 100% and 66% fishmeal (Gomes et al. 1995). Soltan et al. (2008) also showed negative impact on body weight, body length and weight gain in Nile tilapia (*Oreochromis niloticus*) when fishmeal was replaced 100% with cottonseed, canola, sunflower

or sesame meal. The NP-PP group in the present study had higher weight gain compared to NP-FM group and statistically the same as the positive control. Several studies have shown that NP and early larval/juvenile intervention with PP can positively affect growth performance (Kemski et al. 2018; Izquierdo et al. 2015; Balasubramanian et al 2016). Clarkson et al. (2017) reported that early exposure of Atlantic salmon (*Salmo salar*) to wheat gluten-based diet outperformed fish that were never introduced to this PP in their young age. We believe that the NP-PP group was able to achieve higher weight gains compared to the non-programmed group (NP-FM) due to the early intervention of PP leading to an adaptation allowing the fish to utilize PP better in their later growth stages. This confirms most recent study by Kwasek et al. (2020) who reported that zebrafish that were programmed in their juvenile stages with PP (starting at 13 dph) achieved higher weight gains compared to non-programmed individuals. These findings are also concurrent with Fang et al. (2014) who found that zebrafish fed high carbohydrate diets during the first feeding grew better on high carbohydrate diets as juveniles due to upregulation of genes involved in carbohydrate digestion and metabolism. NP has been studied extensively on higher vertebrates and its mechanism has been attributed to epigenetics in ways of DNA methylation patterns, histone covalent and non-coding RNA modifications (Martinez et al. 2012). It is believed that similar epigenetic changes are induced by early exposure to dietary PP in fish (Moghadam et al. 2015). However, in order for NP to work effectively, the programming stage must occur at the right timing in the early development of fish (Kwasek et al. 2020).

Gut microbiome

We hypothesized that NP with PP induces changes in the gut microbiome in larval fish stages and a core microbiota remains within the fish gut throughout its development allowing those same fish respond to PP diet better during the pre-adult stages. The effects of early gut

microbiome intervention can change the physiology and phenotype of the animal later in life (Warne et al. 2019), but it is possible that the gut microbiome will revert back to 'standard' microbiome as has been previously reported in zebrafish (Stephens et al. 2016; Roseleres et al. 2011).

Alpha diversity of gut microbial communities

Dietary formulation, especially plant-based versus fishmeal-based, has been shown to directly influence the gut microbiome composition and consequently fish growth and health performances (Desai et al 2012; Bolnick et al. 2014; Ye et al. 2016). Desai et al. (2012) fed rainbow trout either a fishmeal diet or diets that contained pea, canola, and soybean in form of meals or protein concentrate and found that trout which received a vegetable meal diet presented a higher Shannon's index in the gut microbiome than fishmeal-fed fish. A higher Shannon's index can be a link associated with higher microbiome diversity (Rogers and Aronoff 2016). Baldo et al. (2015) studied five species of closely related cichlids (*Perissodini*) characterized by different feeding behaviours. Even after speciation, all five species presented no differences in the gut alpha diversity. Our study found that throughout the trial, ASVs and Shannon indexes did not show any statistical differences between treatments possibly due to high variability that can be found in fish microbiomes (Stephens et al. 2016).

Beta diversity of gut microbial communities

The present study found that during the first days after hatching (or "pre-dry diet" point) there was a significant difference detected in beta diversity between fish at 4, 6, and 12 dph. At 4 dph, the fish did not receive any food, at 6 dph fish were provided only with rotifers, and at 12 dph the fish received both rotifers and *Artemia* nauplii. The microbial inoculation in the fish gut start from the surrounding water before any food has been consumed from exogenous sources

(Ghanbari et al. 2015). Li et al. (2014) showed that four different species of carp reared in the same water presented different gut microbes between groups, but not within groups, suggesting that species will have similar microbes at hatching and shortly after, possibly explaining why at 4 dph all zebrafish in the current study clustered together strongly. These results also resemble Wilkes et al. (2019) who showed that gut microbiome of yellowtail kingfish (*Seriola lalandi)* had strong clustering as new live food was introduced to the fish. When the fish is young, the gut is mostly colonized by bacteria from the feed, which can be seen in Li et al. (2017) where gibel carp (*Carassius gibelio)* had the same microbiome profile from 13-51 dph when the fish were fed *Artemia* until a pellet was introduced to the diet until the end of the study at 365 dph.

Relative abundance

The gut microbiome of fish can be shifted throughout its life in natural or human selected processes. The fish microbiome goes through many changes from the larval stage to adulthood based on the complexity of the gut. In aquaculture, dietary probiotics and prebiotics are often used to modify the gut microbiome usually to enhance the fish's immune system and to stimulate growth (Vine et al. 2004). However, how and which bacteria are affected seems complex and not fully understood. Providing fish with a different food source can also alter the microbiome (Li et al. 2014). Moreover, the differing types of microbes in the gut can influence the growth potential of fish. Similar fish species can even have the same types of microbes but varying amounts of the same microbe can affect the way the fish grow, and having more of one type of species of bacteria can increase fishes growth performance (Forberg et al. 2016).

Throughout the study different bacteria phyla were detected in zebrafish guts of different ages (4-65 dph). No significant differences were detected in Proteobacteria RA between any of the treatment groups at any of the time points analyzed. However, Proteobacteria were present in

all growth stages even before the zebrafish received their first feed. Proteobacteria is considered to be a part of the zebrafish core microbiome similarly to Fusobacteria, but has also been considered a core phylum of most fish microbiomes (Roeseleres et al. 2011; Givens et al. 2015; Franchini et al. 2014). Fusobacteria was not present at first at the beginning of the larval fish life, but became increasingly common as the fish aged in both NP-PP and NP-FM groups. These bacteria most likely came from the feed, but also over time have been selected to be a part of the fish gut microbiome specifically at 36-65 dph. No differences were detected, however, at any time point in Fusobacteria RA between treatment groups.

Bacteroidetes presented an opposite effect to Fusobacteria in the fish gut, being higher in both NP-PP and NP-FM groups for the first half of the study (up to 24 dph), and then being the lowest in the last two time points at 36 and 65 dph. Furthermore, at 24 dph the NP-PP fish had the highest amount of Bacteroidetes compared to (+) control but not different compared to fish from NP-FM and (-) control. Bacteroidetes and Firmicutes are considered to have an antagonistic relationship meaning if Bacteroidetes increase a decrease in Firmicutes is usually observed and vice versa. This Bacteriodetes:Firmicutes ratio has been used to determine obesity, energy acceptance, and energy retention in higher vertebrates such as mice and humans (Turnbaugh et al. 2006; Ley et al. 2006). This observation has also been made in Common carp (*Cyprinus carpio*) (Li et al. 2013; Li et al. 2017). The RA of Firmicutes did not show any significant differences between treatments throughout the study, but this phylum was generally (numerically) more present in the (+) control and NP-PP groups at the end of PP challenge, while the NP-FM and (-) control groups had almost no populations of Firmicutes present. Firmicutes have many members of beneficial bacteria such as Lactobacillus sp. that are used as prebiotics and probiotics dietary supplements in the aquafeed industry (Suzer et al. 2008). Beneficial

Firmicutes have been found to be high in RA numbers in healthy fish guts, but also in fish that are being fed PP diet (Miao et al. 2018; Dimitroglou et al. 2009). Miao et al. (2018) replaced fishmeal diets at 0, 25, 50 and 75% with SBM for a 63-day feeding trial. All groups that received SBM diet had Firmicutes present, however, when fishmeal was replaced with SBM at 75% Firmicutes decreased. Gajardo et al. (2017) fed Atlantic salmon with five different dietary formulations for 48-days. The fish that received SBM/wheat gluten diet presented higher levels of Firmicutes compared to the fish fed the other four diets which were formulated using animal protein sources. Even though no differences were detected in Firmicutes RA between groups in the present study, it is possible that fish guts in the NP-PP group selected for beneficial Firmicutes similarly to the (+) control that later helped the fish in these treatment groups achieve a higher weight gain compared to both NP-FM group or the (-) control. Nevertheless, this observation remains purely speculative. According to Carmody and Turnbaugh (2012) zebrafish that had higher Firmicutes in their guts presented also higher lipid digestion rate. When gut microbiome shifts were analyzed at different fish ages, the NP-FM group had numerically the highest RA of Firmicutes at 24 dph, but the RA sharply fell from 24 dph, possibly due to lack of adaptation to SBM.

Planctomycetes are a group of bacteria that have been shown to induce negative effects on beneficial gut microbes and have been considered to be an opportunistic pathogenic bacterium to the intestine (Miao et al. 2018; Xiong et al. 2015). The present study found that the (-) control group had the highest RA of Planctomycetes compared to all other groups. At this point, the (-) control fish had been on the SBM diet for 12 days, and it is possible that the negative effects of the SBM were allowing opportunistic bacteria to flourish in the gut. Planctomycetes were also present in all the samples from 4 to 12 dph during in the “live food

stage". However, before the PP challenge, the NP-PP and NP-FM groups had almost no trace of Planctomycetes. Later, however, the NP-PP group showed the highest RA at 65 dph compared to the earlier stages. Although not significant at 65 dph, the NP-FM group presented numerically the highest RA of Planctomycetes possibly suggesting lower "resistance" of this non-programmed group to SBM during PP challenge and consequently imbalance in healthy gut flora. These differences, however, were not significant.

Interestingly, both NP-PP and NP-FM groups presented similar gut microbiome shifts over time although both experienced different feeding regimes. This may be possibly explained by the fact that the gut microbiome of fish seems to be greatly affected at the beginning of the fishes life by the surrounding environment and feed but, as the fish ages and the gut develops, the strong co-evolution of gut microbes and 'fish gut habitat' take over. Wong et al. (2015) showed that zebrafish given a constant feed of either a control (15% fat), high fat (24% fat), or low fat diet (6% fat) for 70 days had similar gut microbiomes at the end of the study, but diverged at the start of the trial. The findings concluded that the fish gut microbiome shifts homogeneously with age no matter what diet was provided to the fish. Yan et al. (2016) studied three different fish species, grass carp *(Ctenopharyngodon idella)*, Chinese perch (*Siniperca chuatsi),* and Chinese largemouth catfish (*Silurus meridionalis)*, and found that over certain key time points of maturation and physiological change, the gut microbiota also been altered in the fish. Similarly, in the present study, as the zebrafish were going through major physiological and morphological changes from larvae (4, 6 and 12 dph) and juveniles (24 dph), to pre-adults (65 dph), the gut microbiome was shifting and accommodating to the more mature intestinal environment.

In Roeseleres et al. (2011) six different locations (five domesticated lab sites and one natural site) were used to determine the 'core microbiome' for zebrafish. Chloroflexi was reported as a major component of the zebrafish 'core microbiome', but was only present at two of the six sites. Pham et al. (2008) also found that Chloroflexi was present in domesticated zebrafish raised in indoor laboratory systems, but not in wild type. In the present study Chloroflexi was the highest between the 'rotifer and Artemia' fed group at 12 dph compared to 'no diet' at 4 dph or rotifer group at 6 dph. When Chloroflexi RA was analyzed overtime, the RA was the highest at 12 dph compared to all other stages in both NP-PP and NP-FM groups. The presence of this phylum was likely derived from live food organisms since it appeared in the "live food stage" (no diet, rotifers, *Artemia* and rotifers), and its presence dropped in the gut microbiome after live food was ended and the dry formulated feed was introduced. Verner-Jeffreys et al. (2003) investigated egg, larvae, juvenile, and adult stages of halibut from three different hatcheries. They found similar results in that certain microbes occurred in higher levels during the live-food *Artemia* stages, and those same microbes almost disappeared during the adult stages when *Artemia* feed was terminated.

CONCLUSIONS

Our hypothesis was that NP with PP induced during the larval zebrafish stage improves growth performance of older fish and that this improved performance induced by NP is possibly associated with modification of the gut microbiota in the larval stages that lead to enhanced growth in pre-adult fish. The programmed group achieved higher weight gains than the control (non-programmed) group confirming again that zebrafish PP utilization can be enhanced by NP. The results, however, also showed no differences between different feeding treatments in fish

microbiota composition at any stage of the trial and consequently, the gut microbiota does not seem to be a mechanism of NP.

The RA of fish gut microbiomes overall did not present any statistical differences throughout the experiment, but the RA of certain phyla such as Chloroflexi, Planctomycetes, and Bacteroidetes presented higher values in some groups at early juvenile stage. The fish gut microbiome also presented differences throughout the fishes life no matter what feed was provided, presenting a natural progression of the zebrafish gut microbiome with age.

CHAPTER 3

PARENTAL PROGRAMMING WITH DIETARY SOYBEAN MEAL AND ITS EFFECT ON THE PROGENY GUT MICROBIOTA IN ZEBRAFISH (*DANIO RERIO*)

ABSTRACT

Nutritional programming (NP) can occur during an organism's life time, but it has also been proven that NP can occur in the parental generation, specifically during embryogenesis. An early stimulus given to parents of an organism could influence the progeny later in life. It has also been shown that there is a strong relationship to parents and offspring in terms of gut microbiome transmission. Therefore, the objectives of this study were: (1) to determine the impact of NP with dietary PP induced during the gonad development on the gut microbiota of broodstock zebrafish; and (2) to assess if the NP of the broodstock has any effect on the gut microbiota and growth performance of the broodstock fish and its progeny. Broodstock fish were fed a commercial diet until two weeks before the spawning date, were broodstock fish were separated and fed either a soybean meal diet or fishmeal diet. When the progeny hatched at 3 days post hatch fish were separated into their experimental tanks. The trial consisted of four different treatments as follows:1. A progeny obtained from FM-fed parents that received FM diet (FMBS-FM, + control);2. A progeny obtained from FM-fed parents that received PP diet (FMBS-PP, - control);3. A progeny obtained from "nutritionally programmed" parents that received PP diet (PPBS-PP);4. A progeny obtained from "nutritionally programmed" parents that received FM (PPBS-FM). This study found that parental programming seemed to have a positive effect on dietary PP utilization in zebrafish, which was reflected by similar growth performance between PPBS-PP, (+) control, and the PPBS-FM progeny groups. Although not significantly different a strong trend was also observed showing numerically higher weight gain between

PPBS-PP and the FMBS-PP groups. Overall, it does not seem that parental NP has an effect on the gut microbiota in offspring. In addition, the evidence seems to suggest that the gut microbiome is likely more influenced by the surrounding environment than the diet provided.

INTRODUCTION

Nutritional programming (NP) is a feeding regime in which early nutritional events alter the physiology of an animal and its response to different dietary regimes later in life (Martinez et al. 2012). NP is triggered during the early stages of an animal's life. However in mammals it has also been shown to have effects as early as embryogenesis in the mother's womb (Oken and Gillmen 2003). Bispham et al. (2003), for example, showed that pregnant sheep (*Ovis aries)* fed at a 40% lower feeding rate had progeny with larger adipose tissue deposits compared to sheep fed to satiation. Sullivan et al. (2010) revealed that mother cattle fed a high protein diet during their first and second trimesters had bull calves with lower concentrations of follicle-stimulating hormone compared to calves that came from mothers fed with a low protein diet, indicating that excess dietary protein reduced the ability of gonad and sex cell formation affecting the bulls fecundity as it ages. Burdge and Lillycrop (2014) showed that different sources of fatty acids given to mice induced a change in DNA methylation specific to the fatty acid desaturase 2 (Fads2) promoter in the liver, and this change was passed down to the progeny of the broodstock.

Although some research on NP and its effects on fish have been demonstrated, the mechanisms behind NP remain vague. NP is believed to be induced through epigenetic changes, which are heritable changes that are not due to changes in DNA sequence. Examples of epigenetic changes include methylation of cytosines in regulatory elements of genes and histone modification, both of which can alter gene expression. These marks can last the whole life of the animal, persist through generations, and change the phenotype of the organism (Moghadam,

Morkore, and Robinson 2015). However, which genes are affected as a result of NP remains unclear (Balasubramanian et al. 2016; Kwasek et al. 2020).

In fish, it has been shown that NP of the broodstock with a different dietary protein or lipid source, could epigenetically influence the fish and affect the number and distribution of osteoblast, myocyte precursor cells, and even gene expression of some growth factors, in the progeny of the broodstock fish (Moghadam, Turid Mørkøre and Robinson 2015). The pathway of parental programming has also been observed in fish, such as Senegalese sole (*Solea senegalensis)*. Morias et al. (2014) tested a control diet (optimal lipid level) and a high lipid diet (specifically in docosahexaenoic acid) and reported that fish that were born from mothers fed a high fat diet had a larger average weight in their juvenile stages compared to fish that were fed the control diet.

There has been a growing strain on some feed ingredients used in fish dietary formulations, particularly marine fish meal (FM) used as protein source, which has been an essential component of most fish diets in the last decades (Gomez et al. 2019). To combat the need for marine source FM, several alternative protein sources have been sought, specifically plant protein (PP) (Booth et al. 2001). Issues with using alternative PP sources, such as soy bean meal (SBM), are evident and include lack of essential amino acids and anti-nutritional factors that impair fish growth and health (Glencross et al. 2019). Studies have shown that higher dietary inclusions of SBM, can have negative effects on fecundity and gametogenesis in adult fish (Callan et al. 2011, Bagheri et al. 2013). Bagheri et al. (2013) revealed that as dietary SBM concentrations increased, egg and sperm count decreased in goldfish (*Carassius auratus).* Callan et al. (2011) found similar interactions with flame angelfish (*Centropyge loriculus*), showing that formulated feeds with increased PP induced lower spawn success. NP in the broodstock and

larval fish with a PP diet has shown to mitigate negative effects that PP can have on the fish (Izquierdo et al 2015; Guerden et al. 2013; Kemski et al. 2018; Kwasek et al. 2020) but the mechanisms of NP and its influence on the gut microbiome and health of fish, are still not well understood.

Microbial transfers between parents and progeny has been observed in a number of invertebrates and non-mammal vertebrates (Funkhouser and Bordenstein 2013). Schmitt et al. (2008) has found that in the phyla Poriferae (sponges) oocytes carry sponge-specific microbes that are passed from generation to generation. Other invertebrates such as deep-water clams (*Calyptogena soyoae)* also carry the chemosynthetic bacteria needed for survival in the oocytes, which are passed down generationally (Carry and Giovanni 1993). The maternal transfer of microbes spans the entire tree of life and includes ray-finned fishes. Sanders et al (2012) has shown that bacteria can be transferred by the oocytes of zebrafish (*Danio rerio)* and passed on to the progeny once breeding occurs. Although the larval zebrafish can acquire some of their microbial makeup from their parents, fish seem to gain large a portion of their gut microbiome during the egg and early larval stages from other sources. These include microbes in the surrounding water, feed once mouths are open, habitat, and several abiotic factors that might affect the bacteria present such as temperature, salinity and more (Tarnecki et al. 2017).

Diet has also been shown to impact the gut microbiota in fish, including reduction in numbers of beneficial microbes, and promotion of growth of deleterious bacteria in the gut. For example, Desai et al. (2012) observed that complete replacement of fishmeal (FM) with low quality plant proteins (PP) such as pea and soybean meals (SBM) had negative effects on rainbow trout (*Oncorhynchus mykiss)* gut microbiota compared to trout that were fed with a FM-based diet. However, there have been other claims indicating that the diet alone does not

necessarily dictate or have a major impact on the gut microbiome (Wang et al. 2016, Lyons et al. 2016). Wang et al. (2016) fed Japanese sea bass (*Lateolabrax japonicus)* diets containing 21, 35 and 42% SBM used as FM replacement and found no change in relative abundance (RA) of bacteria in the gut among the treatments, with Proteobacteria consisting of the majority of the fish gut microbiome. Similar studies such as Lyons et al. (2017) reported that when rainbow trout were fed a diet with 5% microalgae supplementation compared to a control diet containing 100% fish oil, there was no significant difference in overall structure of the gut microbiome between treatments.

Our hypothesis is that a brief exposure of adult (broodstock) fish to a PP diet causes a change in gut microbiome of the adult fish that can then be vertically transferred to the progeny and allow the progeny to better process dietary PP. Therefore, the objectives of this study were: (1) to determine the impact of NP with dietary PP induced during the gonad development on the gut microbiota of broodstock zebrafish; and (2) to assess if the NP of the broodstock has any effect on the gut microbiota and growth performance of the broodstock fish and its progeny.

MATERIALS AND METHODS

The study was conducted in the Center for Fisheries, Aquaculture, and Aquatic Sciences at Southern Illinois University - Carbondale (SIUC) in a recirculated aquaculture system (RAS; Pentair Aquatic Eco-systems, Cary, NC). All experiments were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of SIUC. The SIUC Institutional Animal Care and Use approved all of the protocols performed (protocol# 18-007). During fish handling anesthesia was performed using water bath immersion in tricaine methane sulfonate (MS222) at a recommended concentration, and all efforts were made to minimize pain, stress, and discomfort in the animals.

The RAS system was equipped with 2 mechanical filters, a carbon filter, a biofilter and a UV light. The average water temperature during the experimental period was 26.9± 1.7ºC, the average pH and conductivity were 7.12± 0.52 and 1012±24 S/m, respectively. The photoperiod consisted of 14 hours of darkness and 10 hours of light, with overhead lights on from 8:00-18:00.

Broodstock Rearing and Spawning

Broodstock fish were fed initially with a commercial diet (Otohime C2, Japan). Two weeks before the experiment began, the broodstock were spawned to release any sperm or eggs that were created during the commercial feeding. Broodstock fish were then kept in separate treatment groups and fed 2-3 times a day with FM-based or SBM-based diet. The FM and SBM broodstocks were kept in separate 10 Liter tanks. Both groups were also supplemented with *Artemia* nauplii to ensure proper gonad and egg development, particularly in the SBM group. The fish were fed for two weeks with either a FM or SBM diet to ensure that the gonads, eggs, and sperm were all created with either the SBM or FM protein source. These two weeks of feeding are considered the 'programming stage' for the SBM broodstock.

When it was time to breed, there was a 1:1 ratio of females to males, 10 females and 10 males (Westerfield M 1994). A wire net with a 1.5-millimeter mesh was placed in breeding tank (10 Liter) with a fake plant to induce spawning. The fish were left to breed for 24 hours and then the broodstock were removed. The wire mesh was taken out, and eggs hatched at 27 ºC. At 3 days post hatch (dph) when the majority of the larvae were actively swimming, they were randomly distributed into experimental tanks (3 Liters) for a total of 12 tanks, three replicates per treatment.

Larval zebrafish were fed with saltwater rotifers *Brachionus plicatilis* starting from 3-7 dph. The rotifers were obtained from cysts purchased from a commercial vendor (Brine shrimp

direct, Ogden, Utah). The rotifers were kept in a 30-Liter bucket. The salinity was held at 15 parts per thousand (ppt), the temperature was kept at 23-25 ^{o}C, and constant aeration and light were supplied to the bucket. The rotifers were fed twice a day with powdered *Spirulina* sp. (Earthrise Nutritionals, Irvine, California). The water was changed daily. Zebrafish were then fed with *Artemia* nauplii together with rotifers from 7-10 dph and then just *Artemia* from 10-12 dph. To induce *Artemia* hatching, *Artemia* cysts (GSL brine shrimp, Ogden, Utah) were added to a Macdonald jars and incubated for 24 hours under constant light at 25^{o}C and salinity of 30 ppt. After 24 hours, the *Artemia* hatched into nauplii and were harvested through a 150-micrometer sieve, washed under freshwater, and scooped into the tanks for feeding.

Diet preparation

All the experimental feeds were formulated and produced at SIUC. Two different types of diets were made: a diet made with SBM as a main protein source, and a diet made with FM as a main protein source (Table 3.1). Both FM and SBM diets were formulated to contain 49% protein and 10% lipids. The SBM diet had near 100% FM replacement with 45.5% SBM and 16% soy protein isolate to ensure equal amounts of protein were present in both diets. All dry protein ingredients (FM, krill meal, and SBM) were added to a centrifugal mill, (Zm 100, Retsch Haan, Germany) and ground to 0.5 micrometers. After the centrifugal mill, all ingredients were manually sieved through a .255-micrometer sieve to ensure all particles were of the appropriate and uniform size.

All the dry ingredients (excluding soy lecithin and choline chloride) were added together and mixed for 15 minutes. After all the dry ingredients were mixed the fish oil was added with the soy lecithin dissolved in the oil to ensure even amounts of lecithin throughout the feed. The oil and dry ingredients were mixed again for 15 minutes. After the oil and dry ingredients were

done mixing, water (15% of total mass of feed) was added with choline chloride dissolved in the water to ensure even mixing. Next the feeds were processed using an extruder (Caleva Extruder 20, Sturminster Newton Dorset, England) to produce “noodles”. Feed was slowly added to the extruder at levels between 20-24 Repetitions per minute (RPM) to obtain a proper noodle size. After the noodles were made, they were processed using a spheronizer (Caleva, Sturminster Newton Dorset, England) at 600 RPMs for 3 min, 1800 RPMs for 30 seconds, and then 600 RPMs for 2-5 minutes to finish the process. The noodles were added to the spheronizer to make proper size of uniform spheres for feeding, and to encapsulate the feed to avoid nutrient leeching in water. Finally, the pellets were dried using a freeze dryer (Labconco, Kansas City, MO).

After drying pellets were sieved to appropriate size using a vibratory sieve shaker (Retsch Hann, Germany). The shaker assorted the pellets ending at a powder form (<.155 Micrometers) and starting at the biggest pellet size (>.80 micrometers) with several sizes in between. All finished feeds were stored in bags in -20° C to avoid oxidation. While the feeds were used in experimentation, they were kept in a 4 C^o fridge to keep the integrity of the pellet.

Proximate composition of diets included quantification of the following: crude protein, crude lipid, moisture, and ash. Briefly, samples were analyzed for ash by combustion (550 °C for 5 h) in a muffle furnace (Lindberg Blue M, MA); crude protein (N×6.25) using a Leco nitrogen analyser (Model FP-628, Leco Corporation, St. Joseph, MO); and crude lipid was extracted with chloroform–methanol (2:1, v/v). All dietary samples were analyzed in triplicates.

Experimental Groups and Feeding Regime

The trial consisted of four different treatments as follows:

1. A progeny obtained from FM-fed parents that received FM diet (FMBS-FM, + control);

2. A progeny obtained from FM-fed parents that received PP diet (FMBS-PP, - control);

3. A progeny obtained from "nutritionally programmed" parents that received PP diet (PPBS-PP);

4. A progeny obtained from "nutritionally programmed" parents that received FM (PPBS-FM).

Fish were fed up to satiation until 22 dph. From 22-48 dph, all fish were fed at a restricted rate of 8-9% of the biomass to avoid any discrepancies associated with the feed intake. Restricted rate was adjusted according to the changing biomass and/or feeding activity. Fish were fed until satiation from 3-22 dph to ensure high feed intake, especially of the dry feed, and proper growth during the larval and juvenile stages. After high dry feed consumption was noted restricted feeding was introduced to eliminate potential differences in feed consumption between groups.

Sampling and Measurements

The measured responses that were assessed included: average weight (measured at 20, 27, 34, 41, and 48 dph), weight gain for each treatment group, and gut microbiota diversity and RA (Figure 3.1). Fish were measured on a weekly basis to adjust the feeding rates correctly. Average weight gain was calculated per treatment starting the day of the first weighing till the end of the study. Average weight gain was calculated as follows:

Final mass (g)-initial mass (g)= Weight gain (g)

((Final mass- initial mass)/initial mass) *100= Weight gain (%)

Gut samples were taken throughout the study to assess the structure of the gut microbiome for each treatment. Fish were sampled and frozen directly in liquid nitrogen to preserve the gut microbiome. Pre-NP sampling for the brood fish consisted of taking three fish

from a “common garden” consisting of a 10-liter tank where all fish were kept prior to programming. At the post–program time point three fish were taken from each treatment tank (three from SBM-fed parents, and three from FM-fed parents). Egg samples were taken after spawning from each parental group (SBM and FM), and placed into two 2-milliliter Eppendorf tubes. Similarly, after hatching larvae were sampled from each parental group into three 2-milliliter Eppendorf tubes. The last sampling was conducted when the progeny fish were adults (48 dph) and three fish from each replicate tank were sampled. Refer to Figure 3.1 for a detailed sampling timeline. All samples were stored at -80 C^{o} until analyzed. Water samples were taken at the end of the study (48 dph) in 120 milliliter jars filled to the top. The jars with the water were then placed in a freeze-dryer (Labconco, Kansas City, MO,) for 48 hours, until all water was evaporated. The dried contents as well as all other samples went through the DNA extraction protocol as described below.

DNA Extraction, PCR Amplification, and 16S rRNA amplicon sequencing

Fish were minimally thawed from the freezer on dry ice before dissection. Juvenile fish were dissected, and the intestines were removed from the fish. The intestines were then lysed and the DNA was extracted using protocols of MO BIO Power soil isolation kit (Hilden, Germany). Larval fish and eggs went through the same procedure except they were washed in 70% ethanol for one minute and then washed for 30 seconds under distilled water to remove any attached bacteria. DNA concentrations were quantified using a Qubit fluorometer (ThermoScientific, Waltham MA). Library preparation and sequencing for eggs and larval fish at 3 dph was performed by Shedd Aquarium’s Molecular and Microbial Ecology Group in the A. Watson Armour Center for Animal Health and Welfare (Chicago, IL). Library preparation and

sequencing for pre-programmed and post-programmed adults was conducted by The Roy J. Carver Biotechnology Center (University of Illinois, Champaign-Urbana).

Pre- and post-program adults

Samples were diluted to 2 ng/ul concentrations. A mastermix for amplification was prepared using the Roche High Fidelity Fast Start Kit and 20x Access Array loading reagent according to Fluidigm protocols. For each sample the following reagents were combined :0.5 ul -10X FastStart Reaction Buffer without MgCl2, 0.9 ul -25 mM MgCl2, 0.25 ul DMSO, 0.1 ul -10 mM PCR grade Nucleotide Mix, 0.05 ul -5 U/ul FastStart High Fidelity Enzyme Blend, 0.20 ul water, 1.00 ul Primer V4. Mastermix was aliquoted to a PCR plate. To each well, 1 ul DNA sample and 1 ul Fluidigm Illumina linkers with unique barcode were added.

Bacterial and archaeal DNA was amplified using primer constructs (515f/806rB) targeting the V4 region of the 16S rRNA gene (Walters et al. 2016). All primers were synthesized by IDT Corp. (Coralville, IA). PCR cycling conditions were as follows: one cycle at 50^{o}C for 2 minutes, one cycle of 70 ^{o}C for 20 minutes, one cycle of 95^{o}C for 10 minutes, 10 cycles of 95^{o}C for 15 seconds, 55^{o}C for 30 seconds, and 72^{o}C for one minute, 2 cycles of 95^{o}C at 15 seconds, 80^{o}C for 30 seconds, 60^{o}C for 30 seconds and 72^{o}C for one minute, 8 cycles of 95ºC 15 seconds, 55ºC 30 seconds and 72º 1 minute.

Product was then quantified on a Qubit fluorimeter and stored at -20C. All samples were run on a Fragment Analyzer (Advanced Analytics, Ames, IA) and amplicon regions and expected sizes confirmed. Samples were then pooled in equal amounts according to product concentration. The pooled products were then size selected on a 2% agarose E-gel (Life Technologies) and extracted from the isolated gel slice with Qiagen gel extraction kit (Qiagen)

using a Qiacube robot. Cleaned size selected products were run on an Agilent Bioanalyzer to confirm appropriate profile and determination of average size.

The pool was denatured and spiked with 20% non-indexed PhiX V3 control library provided by Illumina and loaded onto the MiSeq V2 Nano flowcell at a concentration of 6 pM for cluster formation and sequencing. The PhiX control library provides a balanced genome for calculation of matrix, phasing and prephasing, which are essential for accurate basecalling. The libraries were sequenced from both ends of the molecules to a total read length of 250nt from each end. The run generated .bcl files which were converted into demultiplexed compressed fastq files using bcl2fastq 2.20 (Illumina, CA). A secondary pipeline decompressed the fastq files, generated plots with quality scores using FastX Tool Kit, and generated a report with the number of reads per sample/library. The .bcl files were also processed in bcl2fastq 2.20 without demultiplexing and reported. Both sorted and unsorted fastq files were .tgz compressed and posted to a password-secured FTP site.

Larval fish (eggs, 3 dph, and 48 dph)

Bacterial and archaeal DNA was amplified using primer constructs (515f/806rB) targeting the V4 region of the 16S rRNA gene (Walters et al., 2016). The constructs contain Illumina specific adapters followed by 12bp Golay barcodes on each forward primer, primer pads and linkers as well as the template specific PCR primer at the 3' end. PCR was performed in replicate 25µl reactions containing 12.5µl Phusion Hot-Start Flex 2X MasterMix (New England Biolabs), 0.2µM final concentrations of forward primer 515f and reverse primer 806rB, 2µl of template DNA and nuclease free water to equal 25µl. Mock microbial community DNA standards (Zymo Resarch) and negative controls containing no template DNA were prepared with each PCR replicate. Thermal cycling conditions were carried out as follows: 98°C for 30

seconds, 30 cycles at 98°C for 10 sec, 55°C for 30 sec and 72°C for 30 sec, with a final extension of 5 minutes at 72°C. After PCR, replicate amplicons were combined and 5µl of each were electrophoresed in 1.8% agarose gels to confirm amplification of the V4 region. 20µl of each amplicon was then cleaned and normalized using the SequalPrep™ Normalization Plate Kit (Applied Biosystems), and equal volumes of each normalized amplicon were pooled together. The pooled amplicon library was quantified using a Qubit™ 3.0 fluorometer and Qubit™ dsDNA HS Assay Kit (Life Technologies), then further cleaned and concentrated using the UltraClean® PCR Clean-Up Kit (MO BIO Laboratories). The molarity of the pooled library was calculated and was diluted to 2 nM before denaturation and further dilution to a loading concentration of 6 pM. Paired-end sequencing for a total of five hundred cycles was conducted on the Illumina MiSeq platform using custom sequencing primers described previously (Caporaso et al., 2012) with addition of 10% PhiX Control library (Illumina) to increase sequence diversity.

Bioinformatic analysis

For adult fish raw sequence reads were processed using a combination of QIIME2 version 2018.11 Bolyen et al. (2019). Demultiplexed reads were imported into QIIME2 and denoised with DADA2(Callahan et al. 2016) (via q2-dada2), and reads were trimmed at 13 bp and truncated after 230 bp. DADA2 filters sequences for quality, removes chimeric sequences, merges paired-end reads, and produces amplicon sequence variants (ASVs). A rooted phylogenetic tree was then generated using the 'q2-phylogeny' pipeline under default settings, and taxonomy was assigned to ASVs using a Naïve Bayes classifier trained on the SILVA release 132 99% OTUs database (Quast et al. 2012) where sequences had been trimmed to include only the bases within the V4 region bound by the 515F/806R primer pair. Reads that

mapped to chloroplast and mitochondrial sequences were filtered from the sequence variants table using the 'filter_taxa' function.

For larval fish, raw sequence reads were processed using a combination of QIIME2 version 2018.11(Boylen et al.2019) and phyloseq (McMurdie PJ, Holmes S 2013). Demultiplexed reads were imported into QIIME2 and denoised with DADA2(Callahan et al. 2016) (via q2-dada2), and reads were trimmed at 13 bp and truncated after 230 bp. DADA2 filters sequences for quality, removes chimeric sequences, merges paired-end reads, and produces amplicon sequence variants (ASVs). A rooted phylogenetic tree was then generated using the 'q2-phylogeny' pipeline under default settings, and taxonomy was assigned to ASVs using a Naïve Bayes classifier trained on the SILVA release 132 99% OTUs database (Quast et al. 2013), where sequences had been trimmed to include only the bases within the V4 region bound by the 515F/806R primer pair. Reads that mapped to chloroplast and mitochondrial sequences were filtered from the sequence variants table using the 'filter_taxa' function. Data was then imported into phyloseq using the "import_biom" and 'import_qiime_sample_data' functions and merged into a phyloseq object. Samples with less than 3639 reads were discarded, and alpha diversity was calculated using the Shannon index and observed ASVs. The data was then proportionally transformed to a normalized read count of 3639. Beta-diversity was analysed using unweighted UniFrac distances calculated in phyloseq. From these distances, principal coordinates ordinations were calculated and plotted. Taxonomic bar plots were generated using relative abundance from each of the remaining samples.

Statistical analysis

Two-way ANOVAs were run for final average weight and weight gain data, followed by a Tukey's post-hoc test with a significance set at $p<0.05$ using SPSS (Chicago, IL version 25). R

(Boston, MA) version 3.5.2 was used for statistical analysis of data where One-way ANOVAs were generated with the R package multcomp, using a Tukey's post-hoc test with Westfall values for relative abundance (RA) and statistical analysis with a PERMANOVA was used for beta diversity measures. A Faith phylogenetic diversity test was used for adult fish (QIIME 2)

RESULTS

Growth performance and survival

The overall survival of the four treatments was as follows: FMBS-FM 93±2%, PPBS-PP 95 ±1%, PPBS-FM 96 ±1.57%, and FMBS-PP 97% ±1.73. No statistical difference was detected in the survival among the groups (two-way-ANOVA, n=3, 6, 12; $p>0.05$). Table 3.2 present both the final weight gain in grams and percent, and final average weight of fish at the end of the feeding trial. The two-way ANOVA detected a significance for the overall test (n=3 ,6, 12; $p<0.05$), but individual treatments and parents did not have any statistical effect (two-way-ANOVA, n=3,6,12 $p=0.001$).

The PPBS-PP group achieved the same weight gain as the (+) control ($p=0.237$) and the PPBS-FM in terms of grams ($p=0.156$). Although PPBS-PP group achieved weight gains that were numerically higher than the (-) control, significance was also not detected ($p=0.637$). When weight gain was converted into percent weight gain there was no significance detected between any of the groups (two-way-ANOVA n=3.6.12 $p=0.310$). The PPBS-PP had the lowest average weight among the groups except FMBS-PP group. The FMBS-FM and PPBS-FM groups both had the highest average weights compared to PPBS-PP group but not different than FMBS-PP group.

Gut microbiome- Alpha diversity

Alpha diversity was measured at the broodstock stage (dissected intestine), larval stage (3 dph, whole fish; figure 3.2), and at the final time point of the offspring feeding trial (48 dph, dissected intestines; figure 3.3). There was no statistical difference detected in alpha diversity of pre-program and post program broodstock fish (Faith Phylogenetic Diversity, Kruskal-Wallis, df=8, $p>0.05$), larval fish and, or adults at 3 or 48 dph, respectively (Kruskal-Wallis n=2, 3; $p>0.05$).

Gut microbiome - Beta diversity and Relative abundance

Figure 3.4 shows a PCoA plot of the final time point of the study (48 dph). There was no statistical difference between any of the treatments (PERMANOVA, n=12, $p>0.05$). The RA of microbial phyla was analyzed during the following time points: pre-program stage of the broodstock (before NP), the post-programming stage of the broodstock for each set of broodstock (after NP) (Figure 3.5), egg stage (eggs obtained from each broodstock group, Figure 3.6), the larval stage at mouth opening (3 dph; obtained from each broodstock group, Figure 3.7), and of the progeny fish sampled from each treatment group at the end of the study at 48 dph (Figure 3.8).

The majority of the gut microbiome RA for the broodstock zebrafish at pre-programmed stage was Proteobacteria consisting between 45-89% of the RA in fish. All other phyla in the pre-programmed fish appeared at 5% or less which include the phyla Firmicutes, Fusobacteria, Planctomycetes and Tenericutes.

The post-programmed fish had similar gut RA with Proteobacteria consisting of 45-77% of the gut. The SBM broodstock had Acidobacteria comprising 10% of the RA, but all the other phyla (Firmicutes, Fusobacteria, Tenericutes and Verrucomicorbia) consisted of less than 5% of

the RA for the post-programmed SBM broodstock. The main numerical difference between the SBM and FM broodstock at the end of programming was that the FM broodstock had a 33% RA of Tenerictues compared to 0% in the SBM group. No statistical difference however was detected. Like the SBM broodstock, all other phyla FM post-programmed broodstock consisted of less than 5% of the RA.

The eggs originating from both FM and SBM broodstock groups were analyzed for the RA of bacteria inside the egg. The majority of the bacteria that comprised the RA of both groups included: Proteobacteria (62-75%), Fusobacteria (8.2-12%), Bacteroidetes (12-24%). and Patescibacteria (1-3%).

In the analyzed larval fish at 3 dph Proteobacteria (45-75%) and Bacteroidetes *(*23-52%) were the two main phyla present in fish. Fusobacteria Verrucomicrobia, Planctomycetes and Deinococcus-Thermus consisted the rest of the RA.

The guts obtained from fish at the end of the feeding trial at 48 dph consisted mainly of Proteobacteria (22.2-62%) and Fusobacteria (25-75%) with Bacteroidetes (2-22%) and Firmicutes (3-18%) representing the RA of the gut.

Finally, the RA of bacteria in the water was also analyzed from each tank. Proteobacteria had the majority of the tank water RA Firmicutes consisted of 8-22% of the RA, with Actinobacteria consisting of 7-14% of the RA. All other bacteria phyla (Fusobacteria, Planctomycetes, Patescibacteria, Deinococcus-Thermus, Euryarchaeota, Cyanobacteria, Acidobacteria and Verrucomicrobia) represented 5% or less of the RA. No differences were detected between the water 12 different water samples and dominant bacteria phyla ($p=0.48$, one-way-ANOVA, df=11, 3;).

Temporal changes of the gut microbiome

The RA from the guts from post-programmed broodstock (FM-adults/SBM-adults), whole larval fish (3 dph) and final gut samples (48 dph), were all compared over time to detect if there were any differences associated with fish age (Table 3.3). First, the SBM programmed broodstock fish were compared to the progeny they produced when the fish were in larval stage (3 dph) and from the two treatments that were spawned from the SBM-broodstock (PPBS-PP and PPBS-FM). Fusobacteria was significantly the lowest in the SBM-broodstock and larval fish compared to the progeny fish at 48 dph (both treatments PPBS-FM, and PPBS-PP) (one-way-Anova, df=11,3 p=0.002). Bacteroidetes was the highest in larval fish at 3 dph (one-way-Anova, df=11,3 p=0.044) compared to all other fish ages. Proteobacteria was the lowest in fish at 48 dph (both treatments PPBS-FM, PPBS-PP) compared to post-programmed broodstock fish but not different compared to the larval fish (one-way-ANOVA, df=11,3 p=0.034).

The same analysis was conducted for the FM-broodstock fish (Table 3.4). Bacteroidetes was found to be significantly higher in fish at the larval stages (3 dph) compared to post-program FM-broodstock and not different compared to fish at 48 dph (FMBS-FM, FMBS-PP, one-way-ANOVA df=11,3 p=0.018). The only other phyla that was significantly higher in the larval fish compared to all other fish stages was Deinoccoccus-Thermus (one-way-ANOVA, df=11, 3; p=0.0001).

The RA between the water environment and the fish at 48 dph was also analyzed. Overall, there was no statistical difference detected between the microbial phyla of the water and the adult fish except for Fusobacteria. The fish from PPBS-FM group presented significantly higher RA of Fusobacteria in the gut compared to the water environment. Conversely, Fusobacteria was significantly higher in the water environment than in the fish from PPBS-FM

group (one-way-ANOVA, df=11, 3; p=0.035). Finally, the RA of Acidobacteria was higher in the water compared to all treatment at 48 dph(one-way-ANOVA, df=11, 3; p=0.012).

DISCUSSION

Growth performance

The PPBS-PP group were progeny fish that originated from SBM fed parents, and were given a SBM diet throughout the trial. At the end of the trial, our study found that although there was no statistical difference the PPBS-PP group had numerically higher weight gains compared to the FMBS-PP (progeny obtained from FM-fed broodstock that received SBM diet throughout the duration of the study, table 3.2, weight gain column). Kemski et al. (2018) revealed yellow perch that were programmed with SBM had numerically higher weight gains during a 'PP challenge' but were not statistically different than the non-programmed groups. It has been shown that the phenotypic alterations induced by epigenetic changes derived from environmental programming might not be evident at first. For example, Lazzarotto et al. (2016) revealed that when rainbow trout broodstock were programmed with three different diets varying in fishmeal levels (64, 34, and 0%) the progeny did not show any maternal effects from programming at first, before the progeny were exposed to the 'maternal diets', but maternal effects appeared three weeks into the study. Therefore, it is possible that if the present zebrafish trial was extended in time, the epigenetic effects in a form of improved weight gains induced by parental programming would have become more apparent in our adult fish at 48 dph. It is evident that broodstock nutrition impacts the nutritional well-being of the offspring. Seiliez et al. (2017) has shown that when rainbow trout broodstock are fed either a methionine deficient diet (0%, or 0.5%), or a control – methionine containing diet (1.5%) the trout alevins from the methionine deficient group exhibit down regulation of important growth factors. Our study found that the

PPBS-PP group had similar weight gains in terms of grams compared to the two groups that had the highest weight gain and were fed a FM diet. Conversely, the weight gain of FMBS-PP was significantly lower compared to both FMBS-FM and PPBS-FM. Hence, the results suggest that the parental programming had some positive effect on the progeny, allowing the fish in the PPBS-PP group to perform better on PP diet.

It is known that high inclusion of dietary PP can lead to reduced growth in fish as shown by Hansen et al. (2007) who observed that SBM inclusion levels of 50% or higher impairs fish growth. Deng et al. (2016) also showed that as higher levels of soy protein isolate were used to substitute FM in diets at varying replacement levels (0, 25, 50, 75, 87.5 and 100%) specific growth rate decreased in fish fed diets containing of 50-100% soy protein isolate. Furthermore, it has been shown that a PP diet can have negative effects on spawn quality and egg quality of fish. Sink et al. (2010) fed channel catfish broodstock (*Ictalurus puncatatus)* with diets differing in the level and source of protein and lipid (animal vs plant) and found that broodstock fish that received a PP diet supplemented with fish oil suffered in spawn quality and quantity. Similarly, Adewumi et al. (2005) revealed that African catfish (*Clarias gariepinus)* broodstock fed a SBM diet compared to a FM diet suffered in proper ovary and testis formation. Our study found that the PPBS-PP fish average weight was the same compared to FMBS-PP but lower compared to FMBS-FM and PPBS-FM groups. Interestingly, progeny originating from both broodstock groups (FM and PP) that received FM diet all presented the same growth performance (both weight gain and average weight). This could mean that although both broodstock groups were provided with different protein sources (FM or PP) the supplemental feeding with freshly hatched *Artemia* nauplii helped to minimize any nutritional deficiencies by SBM diet that could have affected the gonadal formation, gametogenesis and/or egg development. Although the

effect of NP on gonad quality was not the focus of our study it was observed that both broodstock groups produced viable offspring that was able to perform equally on FM diet. We also believe that greater weight gains and therefore more differences could have been observed if the trial was extended in time. The trial, however, was terminated at 48 dph due to zebrafish achieving sexual maturation and to avoid any discrepancies that the sexual dimorphism between males and females might have had on the growth performance. Perhaps using a species that does not have such a fast-growing rate and a determinate growth would have led to clearer results.

Gut microbiome - Alpha diversity

The whole fish and gut alpha diversity was not significantly different among any of the groups throughout the entire trial in the pre-programmed and post-programmed broodstock, larval stage (3 dph), or the final time point (48 dph The similarity of alpha diversity during the larval stages might possibly relate to their rearing environment since all the fish were occupying the same recirculated system where all abiotic conditions (temperature, pH, salinity and conductivity) were equal in each tank. Due to poor sequence reads, the larval fish could only be tested in duplicate instead of triplicate, which could have also affected the similarity of alpha diversity between the two larval groups. It has been shown that fish of the same species living in the same environment acquire similar gut microbiota profiles (Sylvain and Derome 2017). Sylvain and Derome (2017) found that discus (*Symphysodon aequifasciata)* from 0-21 dph had the same alpha diversity in the gut. Wong et al. (2013) also found that rainbow trout presents a strong 'core microbiome' that does not shift even when fish receive different food sources. In our study at the time of early sampling (3 dph) the fish were at the stage of mouth opening. At this point the zebrafish did not have their first feed yet, which may suggest that the surrounding environment was greatly influencing the developing gut microbiome, and even though the

progeny originated from different broodstocks, the alpha diversities of whole larval fish were similar. Eichmiller et al. (2016) sampled three different wild caught carp species, Common carp (*Cyprinus carpio*), Silver carp (*Hypopthalmicthys molitrix*) and Bighead carp (*Hypopthalmicthys nobilis*), and found that all three shared similar gut microbiome profiles likely due to them sharing the same environment although differences existed in their feeding habits and dietary sources. In our study no differences were detected in alpha diversity in the fish gut at the final time point of the study at 48 dph. Previous studies support findings that when fish receive different diets, alpha diversities of gut microbiota might not be affected (Li et al. 2016). Li et al. (2016) investigated Plateau Pikas (*Ochotona curzoniae)* and noted that different feeds at different elevations did not affect the alpha diversity of the gut microbiome. Bolnick et al. (2014) also found weak associations between alpha diversity of the gut microbiome and formulated diets given to wild and captive three-spined stickle back (*Gasterosteus aculeatus)* and Eurasian perch *(Perca fluviatilis)* showing also that formulated feeds compared to wild natural diet decrease alpha diversity of the gut microbiome in fish. A 'wild' or 'natural' diet would be expected to be characterized by higher alpha diversity due to its microbial variety. Consequently, similar alpha diversities found in the zebrafish study were probably a result of continuous feeding using the formulated "monotonous" feed (FM or PP).

Gut microbiome - Relative Abundance

The relative abundance was analyzed during the pre-program broodstock, post-programmed broodstock, the egg stage, the larval stage, and the final feeding stage for the progeny. Our main focus was to observe if there were any differences in the gut microbiota between the broodstock fish before and after the two-week programming stage, and if that potentially modified parental microbiome would be passed on to the larval fish. It has been well

documented in humans that the passage of gut microbiomes from mother to child occurs during womb development and through the birthing process (Tun et al. 2018). There is some evidence that fish can also 'pass down' gut microbiomes to their offspring. Brown et al. (1997) showed that the bacteria *Flavobacterium psychrophilum* was able to be "transferred" from the mother to the egg in sockeye salmon (*Oncorhynchus nerka)*. Brown et al. (1997) argued that bacteria were most likely transmitted from the ovarian fluid of the female to the surface of the egg. The gut microbiome of fish is not necessarily passed down as it is in mammals but it is determined by selective pressures and host genotype (Yan et al. 2012). Delong (2014) showed that when zebrafish gut microbiome was inserted into a gnobiotic mouse, the mouse gut selected for phyla that were more consistent with a 'standard' mouse gut microbiome, implying that animals have a 'selective gut' in terms of microbes occupying it.

The dominant bacterial phylum found in guts of the broodstock fish, both in the pre- and post-programming stages, was Proteobacteria. Proteobacteria are a major component of the zebrafish gut microbiome as shown by Roeselers et al. (2011). The pre-programmed adult fish also large percentages of Planctomycetes, Fusobacteria and Firmicutes. These bacteria phyla can be considered a part of the core of gut microbiota in fish (Rawls et al. 2016). After the two-week 'programming' stage of the broodstock, Firmicutes was present in the SBM broodstock group but not in the FM broodstock group. Acidobacteria also occurred only in the SBM-fed fish. The only difference in the FM group was the appearance of Actinobacteria, Fusobacteria and Tenericutes, although Tenericutes was present in trace amounts in the SBM group also.

Tenericutes only appeared in the post-programming broodstock and it was not detected in any other whole fish or gut samples. Tenericutes are a bacteria phylum that are likely members of a 'core microbe' in several ray finned fish (Givens et al. 2012) and some cyprinid species (Li

et al. 2016). The gut microbiome has been shown to shift in fish and other vertebrates throughout life (Gerber 2012). Narrowe et al. (2015) exposed fathead minnows (*Pimephales promelas)* to low amounts (100 nanograms/Liter) of triscolan. During the study (2 weeks) the investigators noticed that with or without exposure to triscolan, as the fish matured, the gut microbiome shifted with certain key microbes. This could possibly elucidate why Tenericutes were present in the broodstock fish, but not present in any of the larval fish. It is possible that during those early developmental stages fish did not have the intestinal structure to harbor Tenericutes unlike the older broodstock fish. Rurangwa et al. (2015) used a novel ragworm meal at 85% inclusion rates in diets compared to a diet with 10% ragworm meal and showed that zebrafish from 5-7 dph had completely different gut microbes in both treatments, and suggested that when fish are young, several 'founder species' of bacteria colonize the gut and as the fish ages the gut microbiome selects for species. The Tenericutes Phyla could have also appeared because of the different sequencing methods used between the adult fish and the larval fish.

The very first-time fish come in contact with bacteria is when the egg is laid in the external environment (Egerton et al. 2018). Several fish eggs have a natal defense mechanism against pathogens and bactericidal activity and immunoglobulins available to fight potential infections on the surface of the egg (Hansen and Olafsen 1999). Lectins are molecules that are present in fish eggs that have been shown to exhibit an antibacterial property in invertebrates and are present in cyprinids such as zebrafish; these molecules have also been shown to be selective to certain bacteria (Hansen and Olfasen 1999). However, as mentioned earlier, there have been studies that show that the environment can be a greater factor than the diet in the shaping of the gut microbiome. Bakke et al. (2015) have presented that as cod (*Gauds morhua)* age the gut microbiome changes, but the change of gut microbiota is brought on by the changing and

developing intestinal environment, not the feed given. It has also been noted that although the mother-progeny link is strong in higher vertebrates it is most likely not as relevant in the fish gut microbiome (Sullman et al. 2012). The weak mother-progeny effect in fish, the standardized environment of the tanks, and the eggs not having a fully developed (gut) microbiome might possibly explain why eggs from different treatments harbored the same microbes.

The next time point that was analyzed was the larval stage at mouth opening. Again, Proteobacteria made up a majority of the RA in larval fish from both SBM and FM-fed broodstock. Similarly, to the egg stage, the larvae between both groups shared similar community structure the resemblance of RA is comparable to the trends observed in alpha diversity at that same stage, and can be possibly explained by fish being reared in the same environments. Li et al. (2014) evaluated if paddle fish *(Polyodon spathala)* and Bighead carp had different gut microbiomes if raised in the same pond and given the same feed. The study found differences between species, but reported that within species bacterial communities were similar in terms of RA, showing that similar environments give rise to similar gut microbiome profiles. Furthermore, both larval groups had an influx of Bacteroidetes at this stage even though Bacteroidetes was not present in the broodstock at all. The appearance of Bacteroidetes most likely originated from the surrounding water, which was present in all treatment tanks during the study. Fusobacteria was also present in trace amounts in both larval groups. There were three different groups of bacterial phyla that were numerically different between the larval fish from the SBM broodstock and larval fish from the FM broodstock (both post-programmed) but only appeared in trace amounts. Planctomycetes and Verrucomicrobia were present in the SBM larval group, but not the FM larval group. Interestingly neither of these bacteria phyla were present in the SBM programmed adults. The larval progeny from the FM-broodstock had one bacteria

phylum unique to it being Deinococcus-Thermus. This phylum was not present in the FM programmed broodstock. The presence of these trace phyla Deinococcus-Thermus in the FM progeny and Verrucomicrobia and Planctomycetes appearing in the SBM progeny might be related to their presence in the surrounding water environment. It has been shown that larval cyprinid fish and tilapia (*Oreochromic niloticus)* ingest suspended bacteria in the water column (Beveridge et al. 1991). Since freshwater fish at their early development stage are ingesting these organisms, the bacteria can start to take hold in the gut, especially before the first feeding.

The water environment was also analyzed across all the treatment groups at the study end point (48 dph). It is interesting to note that the RA of the water and fish guts did not differ significantly expect for two phyla, Acidobacteria (between the water and all four treatment groups) and Fusobacteria (between water and the PPBS-FM group). Otherwise, all other bacteria phyla from the water were also found in the fish. Giatsis et al. (2014) has shown that when newly-hatched tilapia are reared in a RAS system, similar microbiomes are found in each tank, but the effect of tank on the gut microbiome is actually low compared to traditional non-RAS systems.

The last samples analyzed were the guts obtained from progeny fish at 48 dph. All four treatments had the similar community structure. All four treatments had four dominant phyla of Proteobacteria, Fusobacteria, Bacteroidetes, and Firmicutes. Overall, there does not seem to be any link between the broodstock NP and gut microbiome diversity in terms of RA.

Temporal changes of the gut microbiome

The study did find some interesting differences in the gut microbiome, specifically between the broodstock and the larval fish. The SBM broodstock fish (post-programmed) shared similar gut community structure with their progeny (SBM larval fish, 3 dph) than either of the

two experimental groups at the end of the feeding trial (PPBS-FM, PPBS-PP at 48 dph) in terms of Proteobacteria and Fusobacteria. The PPBS-FM group and PPBS-PP (48 dph) groups presented lower amounts of Proteobacteria compared to the SBM broodstock group and the larval fish from the SBM broodstock. Fusobacteria had an inverse effect, where the highest percentage of RA was observed in the PPBS-PP and PPBS-FM groups (48 dph) compared to the SBM broodstock and larval fish. Interestingly, the two experimental groups (PPBS-FM, PPBS-PP, at 48 dph) shared similar microbes including Proteobacteria, Fusobacteria, and Bacteroidetes even though the groups were receiving different feeds. Egerton et al. (2020) fed Atlantic salmon 'standard' FM diet and an 80% fishmeal replacement diet (pea protein, soy protein concentrate, and wheat gluten) and found that fish given the PP diet presented different microbial RAs compared to the FM diet-fed fish. Even though there were differences detected, it is important to note that the researchers also found a similar 'core microbiome' between the two experimental groups which consisted of three main families (*Streptococcaceae, Lactobacillaceae* and *Comamonadaceae*) of bacteria contributing to 85% of all the gut microbes in both treatments.

Li et al. (2018) has reported that when male or female herbivorous blunt snout bream (*Megalbrama amblycephla)* and male or female carnivorous Topmouth Culter (*Culter alburnus)* are crossed and hybridized, that the hybrid offspring have a gut-bacterial composition closely related to the blunt snout sea bream more so than the Topmouth Culter, possibly due to morphological shape and function of the gut, but also closer genetic ties to the blunt snout bream. The SBM-broodstock and larval fish shared similar RA more so than the PPBS-FM and PPBS-PP possibly because of the gut microbiome being more malleable at the start of the fish's life to environmental factors, specifically the surrounding water (Talwar et al. 2008). Smith et al. (2015) showed that certain environmental factors such as water and food drive some of the gut

microbiome composition in three spined sticklebacks, but overall the host genetics and host-selection of gut microbes is what determines the majority of the fish gut microbiome especially when the fish ages. This can possibly help explain why the larval fish (3 dph) had some microbes unique to them such as Bacteroidetes being higher compared to the SBM-post-program adults or the two experimental groups at the end of the study (PPPBS-FM, PPBS-PP, at 48 dph).

The same analysis was performed for the post-program FM broodstock , their respective progeny (3 dph) and the two experimental groups (FMBS-FM, FMBS-PP 48 dph). The post-program fish had a similar gut microbiome profile compared to the fish at 48 dph. The differences detected were at the larval stage (3 dph) related to Bacteroidetes which were significantly higher compared to the post-program adults and both groups at 48 dph (FMBS-FM, FMBS-PP), and Deinococcos-Thermus, which were also higher at the larval stage (3 dph) compared to the post-program adults and the two experimental groups. Nayak (2010) has stated that some bacteria are autochthonous, meaning that they stay in the gut because they have an evolutionary filled niche in the gut of the selected animal, while other bacteria are allochthonous, meaning that they fill a niche only for a certain period of time. Perhaps the Deinococcus-Thermus was an allochthonous bacteria that was only present for a short time, but other bacteria, such as Fusobacteria and Proteobacteria are both stable bacteria in the gut as they were similar in the FM-broodstock, larval fish, and both FMBS-FM and FMB-PP groups

CONCLUSIONS

This study found that parental programming seemed to have a positive effect on dietary PP utilization in zebrafish, which was reflected by similar growth performance between PPBS-PP, (+) control, and the PPBS-FM progeny groups. Although not significantly different a strong trend was also observed showing numerically higher weight gain between PPBS-PP and the

FMBS-PP groups. Contrary to other research, it seems that the short exposure (2-weeks) of the broodstock to SBM diet did not have any negative effect on the viability of offspring fish. However, future studies should focus on determining if brief NP as opposed to continuous feeding with PP diet of broodstock fish has a significant impact on gamete (egg and sperm) quality and hatchability rate.

This study also found no differences in alpha diversity between broodstock fish during the pre-program stage and either post-programming stages (FM adults and SBM adults), larval stage, or at the end of the progeny feeding. Furthermore, no differences were detected in the microbiome RA between eggs, larvae, or the guts of broodstock fish and offspring fish at the end of the feeding. Overall, it does not seem that parental NP has an effect on the gut microbiota in fish. In addition, the evidence seems to suggest that the gut microbiome is likely more influenced by the surrounding environment than the diet provided. Future studies should elucidate further the mechanism behind NP to allow for its better utilization as a tool to improve growth performance of fish fed PP-based feeds.

CHAPTER 4

THE EFECT OF EARLY EXPOSURE OF LARVAL LARGEMOUTH BASS (*MICROPTERUS SALMOIDES*) TO DIETARY SOYBEAN MEAL AND SOY SAPONIN ON GROWTH PERFORMANCE AND THE GUT MICROBIOME COMPOSITION

ABSTRACT

Nutritional programming (NP) has been feeding regime that has shown to improve fishes digestion and acceptance of plant protein in a variety of species, but specifically omnivorous fishes. NP has been shown to have similar positive effects on strictly carnivorous fishes, but the effects are not as apparent. It has also been shown that antinutritional factors in some plant proteins, such as saponins in soybean meal are the reason why fish have lower growth performance when given a plant protein diet. These antinutritional factors affect the intestinal structure of the fish, but can also have negative consequences for the gut microbiome of the fish. The objectives of this study were as follows: 1) To assess the effect of NP with formulated SBM diet induced during larval stages on the growth performance of largemouth bass (*Micopterus salmoides)* fed SBM diet in its pre-adult stages; 2) To assess the effect of NP with dietary saponin induced during larval stages on the growth performance of largemouth bass fed SBM diet in its pre-adult stages; 3) To assess the effect of NP with SBM diet or saponin induced in larval largemouth bass on the gut microbiome composition in pre-adult fish. Larval largemouth bass were received at 4 days post hatch (dph) and were distributed at 5 dph into 15 tanks (5 different treatments in triplicate) at densities of 831 (± 98) fish per tank. The trial consisted of five different treatments as follows: 1) A positive control group that received a fishmeal diet throughout the entire trial (positive control; + control); 2) A negative control group that received PP diet throughout the entire trial (negative control; -control); 3) A NP group that received

dietary PP during the larval development followed by fishmeal-based diet during the juvenile stage and PP diet again during a "PP challenge" in the grow-out/pre-adult phase (NP-PP); 4) A fishmeal (FM) group that received fishmeal-based diet during the larval and juvenile stages and was challenged with a PP diet during the grow-out/pre-adult phase (NP-FM); 5) a Saponin group that received a fishmeal-based diet that had saponin added to it (0.3%) during the larval stage, followed by a fishmeal-based diet during the juvenile stage and PP diet during a "PP challenge" in a the grow-out/pre-adult phase (Saponin). NP with SBM or saponin did not improve growth performance of largemouth bass. It also appears that SBM and saponin did not have any effect on the gut microbiome of largemouth bass.

INTRODUCTION

Aquaculture has become the fastest growing animal production sector compared to other livestock due to increasing demand for seafood (Asche et al. 2008). Due to this intensive growth, certain feed components, especially those of marine origin, have become scarcer and more expensive, specifically in fish culture (Olsen et al. 2012). Soybean meal (SBM) and other lower-quality plant protein (PP) sources have been thoroughly investigated as marine fishmeal replacement sources (Hardy 1996). However, SBM, when included at high levels (over 30%) has been shown to induce negative effects on fish including retarded growth and poor health performance reflected by damaged intestinal lining, increased pro-inflammatory cytokine gene expression in the gut, or decreased plasma levels of insulin-like-growth factors and liver mRNA transcripts (Gomez-Requeni et al. 2004; Refstie et al. 2000, Bakke-McKellep et al. 2007, Hedrera et al. 2013; Perera and Yufera 2016). The negative effects of some PP sources, such as SBM, can be attributed to anti-nutritional factors of the meal such as: lectins, protease inhibitors, tannins, and saponins (Yasothai 2016). Bureau et al. (1998) provided Chinook salmon

(*Onocorhynchus tshawytscha)* and rainbow trout (*Oncorhynchus mykiss)* with a 50% fishmeal and 50% SBM diet containing 1% or 0.3% saponin levels, respectively, and found that saponin inclusion at either level induced feed suppression in both fish species, and lowered their weight. Knudson et al. (2008) reported that Atlantic salmon *(Salmo salar)* fed a PP diet (using lupin kernel meal) supplemented with soy saponin, showed severe intestinal enteritis compared to fish fed either a fishmeal control diet or a fishmeal-based diet supplemented with soy saponin. Furthermore, it has also been shown that dietary SBM can decrease the number of microbes found in the fish intestine (Heikkinen et al 2006).

The negative effects of SBM can be counteracted with the use of nutritional programming (NP). NP is a feed regime based on giving a nutritional stimulus early in development that changes the animal's physiology, which can be evoked later in life. Clarkson et al. (2017) programmed Atlantic salmon with a 90% wheat gluten-based diet, and when fish were challenged with wheat gluten diet later in life, the previously programmed fish achieved a 24% higher average weight compared to non-programmed individuals. NP has also been shown to affect the gut microbiome of fish. Geurden et al. (2014) reported that rainbow trout programmed with a high carbohydrate (40% starch, 20% glucose) or a low carbohydrate (20% carbohydrate, 60% protein) diet had similar gut community composition revealing that early exposure of fish to high carbohydrate diets may help maintain bacterial symbiosis within the rainbow trout's intestine.

The objectives of this study were as follows: 1) To assess the effect of NP with formulated SBM diet induced during larval stages on the growth performance of largemouth bass (*Micopterus salmoides)* fed SBM diet in its pre-adult stages; 2) To assess the effect of NP with dietary saponin induced during larval stages on the growth performance of largemouth bass fed

SBM diet in its pre-adult stages; 3) To assess the effect of NP with SBM diet or saponin induced in larval largemouth bass on the gut microbiome composition in pre-adult fish. Our hypothesis was that NP with PP in a form of dry feed induced during the larval stages improves growth performance of pre-adult fish fed PP diet. We also hypothesized that this improved performance induced by NP is likely associated with the modification of the gut microbiota in the early larval stages that lead to enhanced PP digestion, reflected by improved growth.

MATERIALS AND METHODS

The study was conducted in the Center for Fisheries, Aquaculture, and Aquatic Sciences at Southern Illinois University - Carbondale (SIUC). All experiments were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of SIUC. The SIUC Institutional Animal Care and Use approved all of the protocols (IUCAC #18-051) performed. During fish handling, anesthesia was performed using water bath immersion in tricaine methane sulfonate (MS222) at a recommended concentration, and all efforts were made to minimize pain, stress, and discomfort in the animals.

The experiment was carried out using a semi-recirculated aquaculture system with two mechanical (sand) filters (Pentair, Minneapolis) and two bio-filters. The system consisted of 40 (100 L) light blue tanks. The average water temperature was 22.48^{o}C (± 1.11), the pH was 7.70 (± 0.29), and the salinity was kept between 1 and 3 ppt during the live feeding stage to prolong the viability of the live food (Dabrowski and Miller, 2018). The photoperiod consisted of 14 hours of darkness and 10 hours of light, with overhead lights on 8:00-18:00.

Largemouth bass has been chosen as a model species since it is an important commercial, recreational and ecological species as an apex predator throughout the majority of North

America (Heidinger 2000). The largemouth bass is also an important food fish species especially in the live fish markets of the United States and throughout Asia (Tidwell et al. 2003).

Larval distribution and rearing

Fish were received at 4 days post hatch (dph) from the LaSalle Hatchery (Marseilles, IL). When fish arrived, the larvae were incubated in plastic bags set in the system to acclimate the temperature. Water from the system was slowly added to the bag until the fish were acclimated to the system conditions of temperature and pH. Once the fish were acclimated larval bass were placed in a 'common garden'. At 5 dph largemouth bass were distributed into each tank (15 tanks in total, 3 replicates per treatment) volumetrically by taking a 30 mL beaker and filling the beaker to the max volume with larvae. The larvae were then counted out individually; this process was done ten times to get an average of larvae/mL. After that, a 250 mL beaker was used to distribute the larvae to each experimental tank by using the 30 mL beaker average. Fish were distributed to the tanks with an average initial density of 831 (± 98) fish per tank. At 82 dph, the amount of fish in each tank was decreased to only 30 fish per tank. The fish densities were decreased due to limited space in tanks and to reduce any effects on growth potential of the fish and to ensure that feed could be conserved throughout the entirety of the trial.

The largemouth bass were fed with freshly hatched *Artemia* nauplii from 5 dph-25 dph. To induce *Artemia* hatching, *Artemia* cysts (GSL brine shrimp, Ogden, Utah) were added to Macdonald jars and incubated for 24 hours under constant light at 25 ^{o}C and salinity of 30 ppt. After 24 hours, the *Artemia* hatched into nauplii and were harvested through a 150-micrometer sieve, washed under freshwater, and scooped into the tanks for feeding.

Diet preparation - small pellets

All the experimental feeds were formulated and produced at SIUC. Three different types of diets were made: a diet made with SBM as a main protein source (representing a plant-based diet), a diet made with fishmeal as a main protein source, and a saponin supplemented diet made with fishmeal as a main protein source (Table 4.1).

All diets were formulated to be isonitrogenous and isolipidic and contain all essential nutrients at optimal levels required by largemouth bass (NCR, 2011). The level of protein in the diets was 49% and 10% of lipids. The SBM diet had near 100% fishmeal protein replacement with 45.5% SBM and 16% soy protein concentrate to ensure equal amounts of protein were present in both diets. Soy protein isolate was utilized to adjust dietary crude protein level while also leaving room for other ingredients in the formulation, including a minimum level of starch to allow expansion of the diets. All dry protein ingredients (fishmeal, krill meal, and SBM) were added to a centrifugal mill, (Zm 100, Retsch Haan, Germany) and ground to 0.5-micrometers. After the centrifugal mill, all ingredients were manually sieved through a .255-micrometer sieve to ensure all particles were uniform and of the appropriate size.

All the dry ingredients (excluding soy lecithin and choline chloride) were added together and mixed for 15 minutes. After all the dry ingredients were mixed, the fish oil was added with the soy lecithin dissolved in the oil to ensure even amounts of lecithin throughout the feed. The oil and dry ingredients were mixed again for 15 minutes. After the oil and dry ingredients were done mixing, water with dissolved choline chloride (15% of total mass of feed) was added to ensure even mixing. Next, the feeds were processed using an extruder (Caleva Extruder 20, Sturminster Newton Dorset, England) to produce “noodles”. Feed was slowly added to the extruder at levels between 20-24 repetitions per minute (RPM) to obtain a proper noodle size.

After the noodles were made, they were processed using a spheronizer (Caleva, Sturminster Newton Dorset, England) at 600 RPMs for 3 min, 1800 RPMs for 30 seconds, and then 600 RPMs for 2-5 minutes to finish the process. The noodles were added to the spheronizer to make proper size of uniform spheres for feeding, and to encapsulate the feed to avoid nutrient leaching in water. Finally, the pellets were dried using a freeze dryer (Labconco, Kansas City, MO).

After drying pellets were sieved to appropriate size using a vibratory sieve shaker (Retsch Hann, Germany). The shaker assorted the pellets ending at a powder form (<.155 micrometers) and starting at the biggest pellet size (>.80 micrometers) with several sizes in between. All finished feeds were stored in bags in -20° C to avoid oxidation. While the feeds were used in experimentation, they were kept at 4 ^{o}C to keep the integrity of the pellet.

Proximate composition of diets included quantification of the following: crude protein, crude lipid, moisture, and ash. Briefly, samples were analyzed for ash by combustion (550 °C for 5 h) in a muffle furnace (Lindberg Blue M, MA); crude protein (N×6.25) using a Leco nitrogen analyser (Model FP-628, Leco Corporation, St. Joseph, MO); and crude lipid was extracted with chloroform–methanol (2:1, v/v). All dietary samples were analyzed in triplicates.

Diet preparation - large pellets

Prior to mixing, the dry components of the diet were ground to a fine particle size (~0.5mm) using a centrifugal mill (Retsch 2M 100, Haan, Germany). Once the dry components were ground, all ingredients in the diet were mixed (HCM450 Vertical Cutter Mixer, Hobart, Troy, OH) to achieve uniform dispersion. The mixed diets were then run through a food chopper (General Slicing SD-50, General Inc., Weston, FL), and sieved (Retsch AS 200 Basic, Haan, Germany) to obtain a variety of pellet sizes. After sieving, the pellets were dried for 24 hours at

46ºC (Harvest Saver Tray Dryer, Commercial Dehydration Systems Inc., Eugene, OR) to remove moisture from the diets.

Proximate composition of diets included quantification of the following: crude protein, crude lipid, moisture, and ash. Briefly, samples were analyzed for ash by combustion (550 °C for 5 h) in a muffle furnace (Lindberg Blue M, MA); crude protein (N×6.25) using a Leco nitrogen analyser (Model FP-628, Leco Corporation, St. Joseph, MO); and crude lipid was extracted with chloroform–methanol (2:1, v/v). All dietary samples were analyzed in triplicates.

Experimental groups and feeding regime

The trial consisted of five different treatments as follows: 1) A positive control group that received a fishmeal diet throughout the entire trial (positive control; + control); 2) A negative control group that received PP diet throughout the entire trial (negative control; -control); 3) A NP group that received dietary PP during the larval development followed by fishmeal-based diet during the juvenile stage and PP diet again during a "PP challenge" in the grow-out/pre-adult phase (NP-PP); 4) A fishmeal (FM) group that received fishmeal-based diet during the larval and juvenile stages and was challenged with a PP diet during the grow-out/pre-adult phase (NP-FM); 5) a Saponin group that received a fishmeal-based diet that had saponin added to it (0.3%) during the larval stage, followed by a fishmeal-based diet during the juvenile stage and PP diet during a "PP challenge" in a the grow-out/pre-adult phase (Saponin). There were three replicates for each group. All the dietary treatments are presented in Figure 4.1.

Fish were fed up to satiation until 78 dph to ensure high feed intake of formulated diets, particularly during and after the weaning period. Starting at 79 dph fish were fed at a restricted feeding rate as follows: from 78-81 dph fish were fed at a 9% feeding rate, 82-90 dph at 5%, from 90-95 dph at 3%, from 95-100 dph at 2.5%, from 100-117 dph at 2%, and from 118-136

dph fish were fed at 1.5% feeding rate. The feeding rate was calculated biweekly, and whole tank biomass was used to calculate the optimal feeding rate. Between weighing periods feeding rates were established based on assumed biomass based on a FCR of 1, and feeding activity. Fish were fed until satiation during the first 78 days of the study to ensure high feed intake of the formulated diet and proper growth, especially during the larval and juvenile stages. A restricted feeding rate was used after 78 dph to eliminate potential differences caused by variations in feed intake.

Sampling and measurements

The measured responses that were assessed included: final average weight measured at the final day of the study (after PP challenge), weight gain for each treatment, and gut microbial diversity and community composition at four different stages: no food (5 dph), post-programming (26 dph), pre-PP-challenge (84 dph) and at the end of the study (136 dph). Average weight gain was calculated per treatment starting the day of the PP challenge till the end of the study. Average weight gain was calculated as follows:

Final mass (g)-initial mass (g)= Weight gain (g)

Final mass - initial mass/initial mass *100= Weight gain (%)

Larval fish were sampled upon their arrival from a ‘common garden’ at the start of the study before any food was given to the fish. Three 2-milliliter Eppendorf tubes were filled with larval fish and rapidly frozen in liquid nitrogen. In addition, the whole intestinal tracts were sampled from larger size fish (3 fish per tank, 15 tanks total) throughout the study to assess the structure of the gut microbiome for each treatment. Three fish were netted randomly from each tank, padded dry on a paper towel, dissected for only the intestine in sterile conditions, and the

intestines were then frozen directly in liquid nitrogen to preserve the gut microbiome. After that, larval fish and intestines were kept at -80 ºC until the analyses (Figure 4.2).

DNA extraction, PCR amplification, 16S rRNA gene sequencing and amplicon analyses

Larval fish were minimally thawed from the freezer on dry ice and processed whole after an ethanol wash. The intestines dissected from larger fish were then lysed and the DNA was extracted using protocols of MO BIO Power soil isolation kit (Hilden, Germany). Larval fish went through the same procedure except they were washed in 70% ethanol for one minute and then washed for 30 seconds under distilled water to remove any attached bacteria. DNA concentrations were quantified using a Qubit™ fluorometer (ThermoScientific, Waltham MA).

Extracted DNA was brought to the University of Illinois, Roy J Carver Biotechnology Center (Champaign-Urbana, IL) for bacterial 16S rRNA amplification and sequencing. Prior to amplification, samples were normalized to 2 ng/ul concentrations. A mastermix for amplification was prepared using the Roche High Fidelity Fast Start Kit and 20x Access Array loading reagent according to Fluidigm protocols. For each sample the following reagents were combined: 0.5 ul -10X FastStart Reaction Buffer without MgCl2, 0.9 ul -25 mM MgCl2, 0.25 ul DMSO, 0.1 ul -10 mM PCR grade Nucleotide Mix, 0.05 ul -5 U/ul FastStart High Fidelity Enzyme Blend, 0.20 ul water, 1.00 ul Primer V4 (515f/806rB) . Mastermix was aliquoted to a PCR plate. To each well, 1 ul DNA sample and 1 ul Fluidigm Illumina linkers with unique barcode were added.

All primers were synthesized by IDT Corp. (Coralville, IA). PCR cycling conditions were as follows: one cycle at 50ºC for 2 minutes, one cycle of 70 ºC for 20 minutes, one cycle of 95ºC for 10 minutes, 10 cycles of 95ºC for 15 seconds, 55ºC for 30 seconds, and 72ºC for one minute, 2 cycles of 95ºC at 15 seconds, 80ºC for 30 seconds, 60ºC for 30 seconds and 72ºC for one minute, 8 cycles of 95ºC 15 seconds, 55ºC 30 seconds and 72º 1 minute. Product was then

quantified on a Qubit fluorometer and stored at -20C. All samples were run on a Fragment Analyzer (Advanced Analytics, Ames, IA) and amplicon regions and expected sizes confirmed. Samples were then pooled in equal amounts according to product concentration. The pooled products were then size selected on a 2% agarose E-gel (Life Technologies) and extracted from the isolated gel slice with Qiagen gel extraction kit (Qiagen) using a Qiacube robot. Cleaned size selected products were run on an Agilent Bioanalyzer to confirm appropriate profile and determination of average size.

The pool was denatured and spiked with 20% non-indexed PhiX V3 control library provided by Illumina and loaded onto the MiSeq V2 Nano flowcell at a concentration of 6 pM for cluster formation and sequencing. The PhiX control library provides a balanced genome for calculation of matrix, phasing and prephasing, which are essential for accurate basecalling. The libraries were sequenced from both ends of the molecules to a total read length of 250nt from each end. The run generated .bcl files which were converted into demultiplexed compressed fastq files using bcl2fastq 2.20 (Illumina, CA). A secondary pipeline decompressed the fastq files, generated plots with quality scores using FastX Tool Kit, and generated a report with the number of reads per sample/library. The .bcl files were also processed in bcl2fastq 2.20 without demultiplexing and reported. Both sorted and unsorted fastq files were .tgz compressed and posted to a password-secured FTP site.

Raw sequence reads were processed using a combination of QIIME2 version 2018.11 (Bolyen et al. 2019). Demultiplexed reads were imported into QIIME2 and denoised with DADA2 (Callahan et al. 2016) (via q2-dada2), and reads were trimmed at 0 bp and truncated after 250 bp. DADA2 filters sequences for quality, removes chimeric sequences, merges paired-end reads, and produces amplicon sequence variants (ASVs). A rooted phylogenetic tree was

then generated using the 'q2-phylogeny' pipeline under default settings, and taxonomy was assigned to ASVs using a Naïve Bays classifier trained on the SILVA release 132 99% OTUs database (Quast et al. 2012) where sequences had been trimmed to include only the bases within the V4 region bound by the 515F/806R primer pair. Reads that mapped to chloroplast and mitochondrial sequences were filtered from the sequence variants table using the 'filter_taxa' function.

Statistical analysis

One-way ANOVAs were run for final average weight and weight gain data, and to asses the temporal changes of the relative abundance followed by a Tukey's post-hoc test with a significance set at $p<0.05$ (SPSS, Chicago, IL version 25). A Kruskal-Wallis test was used to test the alpha diversity of the Faith's PD test (QIIME2, San Francisco, California). A permANOVA was used to analyze the beta diversity results (QIIME2, San Francisco, California).

RESULTS

Growth performance

At the end of the trial, no differences were detected in the final average weight between the groups ($p=0.149$). Throughout the trial weight gain was analyzed in terms of percent weight gain and in grams. The weight gain was calculated based on the final weigh period (136 dph) and the pre-PP-challenge weighing (84 dph). The weight gain (g and %) of NP-PP group was the same among all other groups. The Saponin group achieved significantly lower weight gain (%) compared to (+) control but not different compared to NP-PP or NP-FM groups. The Saponin and NP-FM groups also achieved weight gain (g) significantly lower compared to the (+) control ($p=.012$) but not different compared to NP-PP group ($p=0.285$) (refer to table 4.2). The (-) control was not included in any of the weight gain data because of severe mortality and

retardation of growth early in the study caused by continuous PP feeding and was ultimately removed from the analyses. No differences were detected in the final average weight between any of the groups.

Survival was also assessed during PP Challenge. The saponin group had a survival rate of 95.60 ±5.09%, and all other treatments (+ control, NP-FM and NP-PP) had 100% survival. The (-) control was excluded midway (110 dph) due to high mortality and overall poor performance caused by continuous PP feeding.

Alpha diversity

Alpha diversity of the gut microbiota was analyzed using Faith's phylogenetic diversity measure. The alpha diversity was assessed at two study points: post-the pre-PP-challenge (84 dph), and at the end of the study (136 dph). The post-programming (26 dph) samples could not be measured because sequence reads had to be trimmed for two samples of the NP-FM group due to poor reads. In both study points the only groups that were analyzed were the NP-PP and the NP-FM. There was no statistical difference detected in the alpha diversity of the gut between the two treatments at any of the two study points (Figure 4.3).

Beta diversity

Beta diversity of the gut microbiota was also analyzed at two study points: at the pre-PP-challenge (84 dph, Figure 4.4 and at the end of the study (136 dph, Figure 4.5). The post-programming (26 dph) samples could not be measured because sequence reads had to be trimmed for two samples of the NP-FM group due to poor reads. At each study point the only groups that were analyzed were the NP-PP and the NP-FM. There was no statistical difference detected in the beta-diversity of the gut between the two treatments at any of the two study points (permANOVA, unweighted unifrac p=0.42).

Community composition

The relative abundance (RA) of microbial taxa for larval and juvenile fish guts was measured at four study points (5, 26, 84 and 136 dph). At 5 dph the larval fish gut microbiome was analyzed while the fish were in a common garden tank. At 26, 84 and 136 dph, guts from three fish from each replicate from the NP-FM and NP-PP treatments were analyzed (Figure 4.6).

At the no food stage (larval stage) the majority of the gut microbial community consisted of Proteobacteria representing 78.95% of the RA. Bacteroidetes consisted of 12.8% of the RA and Acidobacteria comprised 6.14% of the composition. All other phyla (Firmicutes, Fusobacteria, Planctomycetes, Tenericutes and Verrucomicrobia) consisted of 1% or less of the composition at the no food stage.

The analysis of RA at the post-programming stage (26 dph) showed that Proteobacteria was the biggest contributor to the gut microbiome in both treatment groups making up 52.15% and 59% of the NP-PP and NP-FM groups, respectively. Firmicutes consisted of 11.18% of the RA in the NP-PP group but 0% in the NP-FM group. Tenericutes comprised 35.37% of the NP-FM group, but only 4.7% of the NP-PP group. Bacteroidetes included 9.43% of the NP-PP group, but only 1.5% of the NP-FM group. Fusobacteria consisted of 2.6% of the NP-PP group and <1% of the NP-FM group. All other phyla (Actinobacteria, Cyanobacteria, Fibrobacteres) encompassed <1% of the remaining of the RA for both treatment groups.

At the pre-PP challenge point, Fusobacteria accounted for the majority of the RA for the NP-PP and NP-FM groups at 51.39% and 86.62% respectively. Tenericutes contributed 46.12% of the NP-PP group and 9.68% in the NP-FM group. Proteobacteria was the only other bacteria contributing to the RA with 2.6% in the NP-PP group and 3.68% in the NP-FM group.

At the end of the study (136 dph), the majority of the gut microbial community consisted of Fusobacteria at 50.8% and 39.48% in the NP-PP and NP-FM groups, respectively. The NP-PP group consisted of Proteobacteria RA of 37.2%, Tenericutes at 7.7% and Actinobacteria at 3.67%. Firmicutes consisted of <1% of the NP-PP RA. Actinobacteria comprised 29.8% of the NP-FM group, with Tenericutes consisting of 18.18%, Proteobacteria making up 7.22% of the RA, Firmicutes making up 5.26% and Bacteroidetes consisting of <1% of the NP-FM RA.

Temporal changes of the gut microbiome

The temporal changes of the gut microbiome of the seven most abundant phyla were analyzed throughout the four different study points for both the NP-PP and NP-FM groups (Table 4.3 A, B). No statistical differences were detected in any of the phyla except for Proteobacteria in the NP-PP group ($p=0.481$). Proteobacteria were significantly higher at the larval stage compared to the pre-PP Challenge stage (84 dph) ($p=0.034$). However, there were no differences detected in the RA of the gut Proteobacteria between samples from the post-programming (26 dph) and study end point (136 dph) compared to the larval stage or the pre-PP Challenge points ($p=.810$).

The NP-FM group had three phyla that were significantly different throughout the trial: Bacteroidetes, Fusobacteria, and Proteobacteria. Bacteroidetes was higher in the larval stages (5 dph) of the NP-FM group compared to post-programming, pre-PP Challenge and study end point ($p=0.042$). No differences were detected in Bacteroidetes between the other time points (26, 84, 136 dph) ($p= 0.0862$). The RA of the Fusobacteria was lower in both larval (3 dph) and post-Programming stage (26 dph) compared to the pre-PP Challenge (84 dph) ($p=0.012$) and not different with the final study point (136 dph) ($p=0.245$). The RA of Proteobacteria was higher in the larval stage compared to pre-PP Challenge ($p=0.004$) but not different between post-

programming or final study point (p=0.426) The RA at the pre-PP-Challenge was also not different compared to post-Programming or final study point (p=0.872).

DISCUSSION

Growth performance

Several studies have shown that a complete replacement of fishmeal with PP usually leads to retarded growth performance in fish, especially carnivorous species. Espe et al. (2006) revealed that total fishmeal replacement with wheat gluten, corn gluten, or soy protein concentrate in Atlantic salmon (*Salmo salar)* resulted in significantly lower growth rates compared to a 100% fishmeal diet. Hansen et al. (2007) also showed reduced specific growth rates when Atlantic cod *(Gadus morhua)* where given diets with fishmeal replaced at more than 50% with SBM. However, other studies have found that early exposure to PP diet can increase feed intake and improve growth performance of fish fed the same PP diet later in life. For example, Geurden et al. (2013) observed that juvenile rainbow trout programmed with dietary PP presented higher growth rate and feed consumption during PP feeding later in their pre-adult stage compared to trout that had no prior exposure to PP during early development. Kemski et al. (2018) revealed that yellow perch (*Perca flavescens*) juveniles when programmed with almost complete SBM diet achieved a higher weight gain percent than the non-programmed fishmeal and wheat gluten-based diet fed fish when all fish were "challenged' with a SBM diet later in life. It is important to note, that Kemski et al. (2018) did not obtain significant results, however, the strong numerical trends in growth data may suggest that the NP with SBM has potential to improve growth of this species.

Although several past studies have revealed that NP seems to be a feasible approach to improve PP utilization in carnivorous fish, we found that the NP induced in larval largemouth

bass using SBM diet did not improve growth performance of the fish in pre-adult stages. Perera and Yufera (2016) programmed zebrafish with SBM or soy protein concentrate-based diets when zebrafish first reached mouth opening at 3 days post fertilization and found no significant differences in terms of growth between programmed and non-progammed zebrafish later in their life. Similarly, Zambonino-Infante et al. (2019) reported that European sea bass (*Dicentrachus labrax)* programmed with a high carbohydrate diet (34%) at mouth opening (6 dph) did not achieve higher weight gain compared to non-programmed fish. Conversely, Kwasek et al. (2020) exposed zebrafish to SBM diet at 13 dph, and the programmed fish achieved a higher weight gain than non-programed individuals right before reaching sexual maturation. This might suggest that the timing of NP is critical. We attempted to program largemouth bass at 8 dph, shortly after mouth opening, and during the first two weeks the fish received SBM as part of a formulated dry diet (8-22 dph) as well as *Artemia* nauplii used as supplementary feed to ensure high survival. It is reasonable to assume, that at the young stage the feed intake of the live food was probably greater as opposed to the dry PP diet. It is well-known that larval stages of many fish species do not ingest dry feeds easily as the first food (Dabrowski 1984, Ayele et al. 2015 Kemigabo et al. 2015).

Consequently, low SBM diet ingestion during the larval stage would not expose the fish to sufficient quantities of SBM (as well as saponin) possibly confounding the results achieved during the PP Challenge. This could also confirm why some of the previous NP studies were unsuccessful (Perera and Yufera 2016; Zambonino-Infante 2019). Perhaps if the NP stimuli (SBM in dry formulated diet form) was introduced at a later stage when fish where fully metamorphosed or in a different form (SBM-enriched *Artemia* nauplii) the NP effect in the NP-PP group would have been more pronounced as the fish aged.

Our study also found that the Saponin group achieved significantly lower weight gain compared to (+) control but not different compared to NP-PP or NP-FM groups. Based on previous studies it is evident that as the level of saponin derived from SBM in a diet increases, certain important quantified production parameters such as: weight gain, feed efficiency ratio, and apparent digestibility coefficient, decrease (Chen et al. 2011). Soy saponins are known to be associated with negative impact on fish health, specifically with intestinal inflammation on both molecular and histological level (Francis et al. 2001; Molinari et al. 2020). Hedrera et al. (2013) reported that dietary saponin inclusion at 3.3% in zebrafish diet led to the highest rate of intestinal inflammation. We hypothesized that programming the fish with dietary saponin would potentially help the fish utilize SBM-based diet better through adaptation of the gut to this anti-nutritional factor. The results, however, did not show any significant impact of early exposure to dietary saponin on later growth of largemouth bass on PP diet. SBM is characterized by many other antinutritional factors (lectins, tannins, trypsin inhibitors, etc.) that can be attributed to intestinal damage in fish (Refesti et al. 1998). Our study, however, does not indicate that saponin and/or saponin adaptation is the "driver" behind the NP and the often-associated positive response to SBM diet.

Alpha diversity

The alpha diversity was analyzed for the two study points: pre-PP-challenge, and at the end of the study. Only two groups were analyzed, the NP-PP group and the NP-FM group at these time points. There were no significant differences detected between any of these three groups at any time point (84 or 136 dph) in terms of gut microbiome alpha diversity. Similarly, no differences were found in gut beta diversity between NP-FM and NP-PP groups. Yan et al. (2016) has showed that the host fish has a bigger impact on the gut microbiome assemblage then

the environment. These investigators took three different species of fish, herbivorous grass carp (*Ctenoparyngodon idellus,)* and two carnivorous species *(Siniperca chuasti, Silurus meridionalis)* and raised them all in a common water environment in net cages. The study revealed that although all species were reared in the same water environment, the gut microbiome alpha diversity was similar within the same species, but different between the three fish species analyzed. A similar effect was observed in our study, where largemouth bass were reared in identical water environments in a semi-recirculated system were pH, temperature, and conductivity were all kept constant, and all the treatments presented similar alpha diversity although the groups went through different feeding regimes. Although our study did not investigate two different species of fish, the different feeding regimes of the same species yielded similar results between the NP-FM and NP-PP groups throughout the trial. Wu et al. (2012) has revealed that grass carp *Ctenopharyngodon idella* raised in completely different water environments still assembled the same 'core gut microbiome', showing once again that the host interactions and the gut selection of microbes seem often more influential than the environmental change. Since the water environment in all treatment tanks was standardized, and the only variable in the study was the feed type given, it appears that protein source (marine or plant) and therefore, different dietary formulations, did not affect the gut microbiome significantly in terms of the alpha diversity. In addition, it also appears that the NP did not have an effect on the alpha diversity. Benson et al. (2010) observed that certain quantitative trait loci are conserved in the genome of the mice, and can even be broad range or litter-specific to the mouse, giving mice a core microbiome of 64 specific species. It has also been reported that certain fish species, such as the Trinidad guppy *(Poecilia reticulata),* have core microbiomes that are not associated with the ecotype, clearly showing a strong genetic linkage to the host (Sullman et al. 2015).

Beta diversity

Our study found no differences in beta diversity between treatments even though different feed formulations were provided to the fish. Llewellyn et al (2015) studied the gut microbiome diversity (PcoA, Pairwise unweighted unifrac distances) in Atlantic salmon throughout the fresh and saltwater life stages. Although significant differences were found between the life stages, salmon within the same life stage from both environments did not differ in gut microbiome diversity. This clearly indicates that the environment might not always be the main contributor to the gut microbiome in fish, but that the host is selecting for certain microbes. Smith et al. (2015) observed 10 geographically different populations of three-spined sticklebacks *Gasterosteus aculeatus* and revealed that the different geography of the stickleback fish caused an alteration in beta diversity of these fish. Under further investigation however, it was concluded that the changes of gut RA were most likely due to host selection and genetics and not the water source.

Community composition and temporal shifts of the gut microbiome

Our study found that at the start of the trial, the dominant taxa of the gut microbiome were Proteobacteria, Bacteroidetes and Acidobacteria. This is in agreement with previous studies which reported these three phyla to be common in largemouth bass and considered to be a part of the largemouth bass 'core gut microbiome' (Larsen et al. 2014). Proteobacteria in the NP-PP group was the only phyla of bacteria that presented significant differences. The RA of Proteobacteria was significantly higher at the larval stage compared to the pre-PP Challenge stage. However, there were no differences detected in the RA of the gut Proteobacteria between samples from the post-programming and study end points compared to the larval stage or the pre-PP Challenge point. Similarly, in the NP-FM groups, the RA of Proteobacteria was higher in

the larval stage compared to pre-PP Challenge but not different between post-programming or final study point. Lin et al. (2020) found that largemouth bass fed different levels of dietary fiber (4 or 8%) originating from SBM presented higher RA of Proteobacteria. This could possibly explain why the higher Proteobacteria occurred when the fish were receiving a SBM diet (at the end of the study), and why Proteobacteria RA dropped numerically in the NP-FM group when fishmeal-based diet was introduced. This could also possibly explain why RA of Proteobacteria decreased when fishmeal was fed to the NP-PP group during the pre-PP-challenge phase. The different sampling methods, (whole fish for larvae, intestinal tract for juveniles) could also be a reason for differences in RA of certain microbes.

Bacteroidetes was also a phylum that had significant temporal changes, but only in the NP-FM group. In the larval stages, Bacteroidetes consisted of 12% of the gut microbiome RA, and completely dwindled to almost 0% at the end of the trial. A similar trend, although not significant, can be seen in the NP-PP fish. Bacteroidetes has been shown to make up very little RA in the adult largemouth bass gut microbiome community composition (Larsen et al. 2014), but it still appeared to be a major component at the start of the fish life. Burns et al. (2015) have noted that time and developmental stage are major contributors to fish gut microbiome assemblage in zebrafish (*Danio rerio)*.

The last bacterial phyla that changed over time significantly were Fusobacteria in the NP-FM group. The RA of the Fusobacteria was lower in both larval and post-Programming stage compared to the pre-PP Challenge and not different with the fish from the final study point (136 dph). This again appears to be associated with the gut microbiome specific selection throughout the different fish developmental changes which has been noted by several studies on zebrafish (Wong et al. 2012, Stephens et al. 2016).

CONCLUSIONS

Our study found that NP with SBM diet or dietary saponin during the larval stages did not improve PP utilization and growth performance of largemouth bass in its pre-adult age. Perhaps NP using live food-enriched with SBM would ensure high feed intake or earlier termination of *Artemia* nauplii feeding during larval bass culture, which may force the fish to ingest the dry PP feed as opposed to live food. This could be a more feasible approach to successful early exposure to PP.

Our study also found that NP with SBM diet or dietary saponin did not have significant effects on the largemouth bass gut microbiome, and there does not seem to be any gut microbiome modification associated with the NP. It seems, however, that the water environment as well as the host might play important roles in shaping the gut microbiome. Those environmental and genetic factors appear to out-weigh the influence of the feed provided. This speculation, however, needs to be further investigated. Specifically, the genetic factors that influence the gut microbiome and how that gut microbiome of certain species is selected should be investigated.

CHAPTER 5

GENERAL CONCLUSIONS

- NP induced in larval zebrafish leads to improved dietary PP utilization in zebrafish in pre-adult stages. However, parental NP does not seem to improve growth performance in zebrafish progeny.

- NP induced in larval largemouth bass with dry formulated PP-based feed did not significantly improve fish growth in pre-adult stages. Lack of significant results was likely associated with poor feed intake of the dry diet during the first weeks after hatching. Further research should consider evaluation of the optimal timing for NP as well as methods for delivery of SBM (i.e. SBM-enriched live food) to allow for most efficient exposure to this PP and consequently better adaptation of the fish to this dietary ingredient.

- NP with SBM-based diet did not have any significant effect on the zebrafish or largemouth bass gut microbiome, and there does not seem to be any gut microbiome modification associated with the NP.

- Zebrafish and largemouth bass appear to have a strong ‘core microbiome’ and dietary shifts do not seem to influence the established microbiome profile for a given species. Future studies should focus on evaluation of different formulations perhaps solely based on plants (protein and oil) as well as other plant ingredients.

- The NP feeding regime has potential to guide the industry into using lower-quality PP sources, leading to more sustainable and cost-effective industry. However, further research should be conducted on the actual mechanism of NP, as it remains vague.

- Studies on both zebrafish and largemouth bass presented major shifts in the gut microbiome as the fish aged. In addition, the core microbiomes of both species appear to become more pronounced as the fish become adults. There seem to be an evolutionary tie between host and its gut microbiome. More studies, however, should further investigate this and the genetic effects on gut microbiota development and its heritability.

- The findings from these studies present zebrafish as a good model species for largemouth bass and potentially other fish species of commercial importance. This is based specifically on the gut microbiome development patterns and the 'core microbiome' present in both species. The zebrafish model should be taken with caution, however, due to its fast-generational time and determinate growth, which can lead to early study termination.

EXHIBITS

TABLES

Table 2.1. Feed formulation and its proximate composition (g/100g).

Ingredient	FM	SBM
Fish Meal[1]	63.8	-
Soybean Meal[2]	-	46.3
Soy Protein Isolate[3]	-	15.4
Krill Meal[4]	10.0	10.0
CPSP[5]	5.8	5.7
Dextrin	5.3	-
Fish Oil[6]	3.9	7.1
Soy Lecithin[7]	4.7	4.7
Mineral Mix[8]	2.4	2.4
$CaHPO_4$	-	1.4
Vitamin Mix[9]	2.0	2.0
Vitamin C[10]	0.1	0.1
Choline Chloride	0.1	0.1
Methionine	-	0.5
Lysine	-	2.3
Threonine	-	0.1
Taurine	0.9	0.9
Guar Gum	1.0	1.0
Sum	**100**	**100**
Analyzed composition		
Crude protein (N x 6.25)	54.51 ± 0.57	53.30 ± 0.13
Crude lipids	17.25 ± 0.47	16.89 ± 0.08
Ash	15.39 ± 0.09	9.10 ± 0.27

[1] Mechanically extracted menhaden meal, stabilized with 0.06% ethoxyquin (Omega Protein, Reedville, VA, USA).
[2] Solvent extracted soybean meal (Premium Feeds, Perryville, MO, USA).
[3] Crude protein concentration min. 92% (Dyets Inc, Bethlehem, PA, USA).
[4] Proccesed *Euphausia superba* (Florida Aqua Farms, Dade City, FL, USA).
[5] Soluble fish protein hydrolysate (Sopropeche S.A., Boulogne Sur Mer, France).
[6] Cod liver oil (MP Biomedicals, Solon, OH, USA).
[7] Refined soy lecithin (MP Biomedicals, Solon, OH, USA).
[8] Bernhart-Tomarelli mineral mix with 5ppm selenium in a form of sodium selenite (Dyets, Bethlehem, PA, USA).
[9] Custom Vitamin Mixture (mg/kg diet) Thiamin HCl, 4.56; Riboflavin, 4.80; Pyridoxine HCl, 6.86; Niacin, 10.90; D-Calcium Pantothenate, 50.56; Folic Acid, 1.26; D-Biotin, 0.16; Vitamin B12 (0.1%), 20.00; Vitamin A Palmitate (500,000 IU/g), 9.66; Vitamin D3 (400,000 IU/g), 8.26; Vitamin E Acetate (500 IU/g), 132.00; Menadione Sodium Bisulfite, 2.36; Inositol, 500 (Dyets, Bethlehem, PA, USA).
[10] L-Ascorbyl-2-Polyphosphate (Argent Aquaculture, Redmond, WA, USA).

Table 2.2. Weight gains throughout the PP-challenge in grams and percent The NP-FM groups is the non-programmed group, and the NP-PP group is the programmed group. Different letters indicate a statistical difference with standard deviation (weight gain grams p=0.01,weight gain % p=0.02).

Treatment	Weight gain (g)	Weight gain (%)	Average weight (g)
- Control	0.119^{a} (±0.009)	189^{a} (± 3.69)	0.145 (± 0.010)
+ Control	0.200^{bc} (±0.030)	271^{bc} (± 35.5)	0.230 (± 0.030)
NP-FM	0.173^{c} (±0.009)	229^{c} (± 7.85)	0.199 (± 0.010)
NP-PP	0.201^{b} (±0.008)	279^{b} (± 17.9)	0.228 (± 0.009)

Table 2.3. Relative abundance (%, with standard deviation) of the top 6 bacteria phyla in the zebrafish gut microbiome throughout the entire study in the NP-FM (non-programmed group) and NP-PP (programmed group) groups. Different letters indicate significance ($p<0.05$).

NP-FM	6 dph	12 dph	24 dph	36 dph	65 dph
Proteobacteria	56.95^{a}(±2.23)	37.93^{ab}(±5.00)	30.18^{bc}(±17.97)	29.23^{b}(±7.51)	52.26^{ab}(±0.19)
Fusobacteria	0.07^{a}(±0.04)	0.06^{a}(±0.03)	11.44^{a}(±8.25)	58.30^{b}(±12.73)	38.64^{b}(±10.72)
Bacteroidetes	31.82^{a}(±11.29)	23.23^{abc}(±10.08)	43.12^{a}(±8.53)	3.63^{b}(±0.96)	0.26^{bc}(±0.23)
Firmicutes	1.14(±0.93)	0.17(±0.10)	14.78(±16.63)	5.56(±1.19)	0.38(±0.39)
Chloroflexi	6.94^{a}(±10.96)	34.11^{b}(±10.61)	0.17^{a}(±0.29)	0.16^{a}(±0.17)	0.08^{a}(±0.11)
Planctomycetes	0.36(±0.40)	0.31(±0.06)	0.08(±0.07)	0.38(±0.28)	4.39(±5.57)

NP-PP	6 dph	12 dph	24 dph	36 dph	65 dph
Proteobacteria	56.95(±2.23)	37.93(±5.00)	49.06(±18.30)	24.45(±9.96)	47.32(±29.12)
Fusobacteria	0.07^{a}(±0.04)	0.06^{a}(±0.03)	0.05^{a}(±0.04)	52.86^{b}(±10.56)	46.28^{b}(±38.78)
Bacteroidetes	31.82^{a}(±11.29)	23.23^{ab}(±10.08)	47.20^{a}(±19.37)	9.00^{b}(±7.24)	0.29^{b}(±0.26)
Firmicutes	1.14^{a}(±0.93)	0.17^{a}(±0.10)	1.19^{a}(±0.44)	12.96^{b}(±7.05)	4.41^{b}(±1.45)
Chloroflexi	6.94^{a}(±10.96)	34.11^{b}(±10.61)	0.57^{a}(±0.80)	0.05^{a}(±0.01)	0.20^{a}(±0.06)
Planctomycetes	0.36^{b}(±0.40)	0.31^{b}(±0.06)	0.70^{b}(±0.32)	0.50^{b}(±0.09)	1.64^{a}(±0.25)

Table 3.1. Feed formulation and its proximate composition (g/100g).

Ingredient	FM	SBM
Fish Meal[1]	63.8	-
Soybean Meal[2]	-	46.3
Soy Protein Isolate[3]	-	15.4
Krill Meal[4]	10.0	10.0
CPSP[5]	5.8	5.7
Dextrin	5.3	-
Fish Oil[6]	3.9	7.1
Soy Lecithin[7]	4.7	4.7
Mineral Mix[8]	2.4	2.4
$CaHPO_4$	-	1.4
Vitamin Mix[9]	2.0	2.0
Vitamin C[10]	0.1	0.1
Choline Chloride	0.1	0.1
Methionine	-	0.5
Lysine	-	2.3
Threonine	-	0.1
Taurine	0.9	0.9
Guar Gum	1.0	1.0
Sum	**100**	**100**
Analyzed composition		
Crude protein (N x 6.25)	54.51 ± 0.57	53.30 ± 0.13
Crude lipids	17.25 ± 0.47	16.89 ± 0.08
Ash	15.39 ± 0.09	9.10 ± 0.27

[1] Mechanically extracted menhaden meal, stabilized with 0.06% ethoxyquin (Omega Protein, Reedville, VA, USA).
[2] Solvent extracted soybean meal (Premium Feeds, Perryville, MO, USA).
[3] Crude protein concentration min. 92% (Dyets Inc, Bethlehem, PA, USA).
[4] Proccesed *Euphausia superba* (Florida Aqua Farms, Dade City, FL, USA).
[5] Soluble fish protein hydrolysate (Sopropeche S.A., Boulogne Sur Mer, France).
[6] Cod liver oil (MP Biomedicals, Solon, OH, USA).
[7] Refined soy lecithin (MP Biomedicals, Solon, OH, USA).
[8] Bernhart-Tomarelli mineral mix with 5ppm selenium in a form of sodium selenite (Dyets, Bethlehem, PA, USA).
[9] Custom Vitamin Mixture (mg/kg diet) Thiamin HCl, 4.56; Riboflavin, 4.80; Pyridoxine HCl, 6.86; Niacin, 10.90; D-Calcium Pantothenate, 50.56; Folic Acid, 1.26; D-Biotin, 0.16; Vitamin B12 (0.1%), 20.00; Vitamin A Palmitate (500,000 IU/g), 9.66; Vitamin D3 (400,000 IU/g), 8.26; Vitamin E Acetate (500 IU/g), 132.00; Menadione Sodium Bisulfite, 2.36; Inositol, 500 (Dyets, Bethlehem, PA, USA).
[10] L-Ascorbyl-2-Polyphosphate (Argent Aquaculture, Redmond, WA, USA).

Table 3.2. Growth performance data obtained at 48 dph (end of the progeny feeding). The groups are as follows: 1) FMBS-FM, progeny from FM broodstock given FM; 2) FMBS-PP, progeny from FM broodstock given PP; 3) PPBS-FM, progeny from PP broodstock given FM; 4) PPBS-PP, progeny from PP broodstock given PP. Different letters indicate a statistical difference between groups ($p<0.05$).

Treatment	Weight gain (g)	Weight gain (%)	Average weight (g)
FMBS-FM	0.157^{a} (±0.029)	1281.27 (± 298.17)	0.170^{a} (±0.032)
FMBS-PP	0.090^{b} (±0.028)	745.84 (± 459.84)	0.130^{ab} (±0.023)
PPBS-FM	0.164^{a} (±0.010)	1289.81 (± 20.54)	0.177^{a} (±0.011)
PPBS-PP	0.114^{ab} (±0.028)	852.50 (± 452.53)	0.104^{b} (±0.025)

Table 3.3. Temporal shifts of the gut microbiome relative abundance (RA, %, standard deviation) at different fish age stages: broodstock fish after NP, larvae (3 dph), and young adults (48 dph, PPBS-FM stands for progeny from PP broodstock given FM, and PPBS-PP stands for progeny from PP broodstock given PP). All fish stages presented originate from the SBM-fed adults. Different letters indicate statistical significance ($P<0.05$) based on one-way ANOVA.

Phyla	**Post-Program SBM Adults**	**Larvae**	**PPBS-FM**	**PPBS-PP**
Firmicutes	1.82 ± 9.18^{a}	0 ± 0.00^{a}	9.33 ± 9.29^{b}	8.27 ± 5.30^{b}
Fusobacteria	3.62 ± 0.23^{a}	5.47 ± 7.07^{a}	53.56 ± 3.61^{b}	50.93 ± 0.01^{b}
Proteobacteria	94.28 ± 28.01^{a}	46.50 ± 2.12^{ab}	34.42 ± 11.53^{b}	27.87 ± 5.29^{b}
Bacteroidetes	0 ± 0.00^{a}	44.5 ± 7.78^{b}	$10.67\ 6.42^{a}$	7.67 ± 3.06^{a}
Planctomycetes	0 ±0.00	1.52 ±1.41	0 ±0.00	0 ±0.00
Verrucomicrobia	0 ±0.00	1.12 ±1.42	0 ±0.00	0 ±0.00

Table 3.4. Temporal shifts of the gut microbiome relative abundance (RA, %) at different fish age stages: broodstock fish after NP, larvae (3 dph), and young adults (48 dph, FMBS-FM are progeny from FM broodstock fed FM, FMBS-PP are progeny from FM broodstock fed PP). All fish stages presented originate from the FM-fed adults. Different letters indicate statistical significance ($P<0.05$) based on one-way ANOVA.

Phyla	**Post Program FM Adults**	**Larvae**	**FMBS-FM**	**FMBS-PP**
Firmicutes	0.20 ±0.35	0 ±0.00	9.33 ±7.77	14.54 ±19.80
Fusobacteria	18.40 ±30.83	1.50 ±2.12	42.67 ±16.62	37.67 ±1.41
Proteobacteria	46.14 ±49.06	71.50±4.94	34.67 ±16.77	26.67 ±4.94
Tenericutes	32.70 ±56.55	0 ±0.00	0±0.00	0 ±0.00
Bacteroidetes	0 ±0.00[a]	23.50±2.12[b]	5.33 ±2.52[ab]	9.00 ±13.44[ab]
Deinococcus-Thermus	0 ±0.00[a]	2.52±0.71[b]	0 ±0.00[a]	0 ±0.00[a]

Table 4.1. Feed formulation and its proximate composition (g/100g).

Ingredient (%)	FM Diet	SBM Diet	Saponin
Fish Meal[1]	63.8	-	63.7
Soybean Meal[2]	-	46.3	-
Soy Protein Isolate[3]	-	15.4	-
Krill Meal[4]	10.0	10.0	10.0
CPSP[5]	5.8	5.7	5.0
Dextrin[3]	5.3	-	5.1
Fish Oil[6]	3.9	7.1	4.2
Soy Lecithin[6]	4.7	4.7	5.0
Mineral Mix[3]	2.4	2.4	2.5
$CaHPO_4$[7]	-	1.4	-
Vitamin Mix[3]	2.0	2.0	2.0
Vitamin C[8]	0.1	0.1	0.1
Choline Chloride[3]	0.1	0.1	0.1
Methionine[3]	-	0.5	-
Lysine[3]	-	2.3	-
Threonine[3]	-	0.1	-
Taurine[3]	0.9	0.9	1.0
Guar Gum[3]	1.0	1.0	1.0
Saponin	-	-	0.3
Total	100.0	100.0	100.0

[1] Mechanically extracted menhaden meal, stabilized with 0.06% ethoxyquin (Omega Protein, Reedville, VA, USA).
[2] Solvent extracted soybean meal (Premium Feeds, Perryville, MO, USA).
[3] Crude protein concentration min. 92% (Dyets Inc, Bethlehem, PA, USA).
[4] Proccesed *Euphausia superba* (Florida Aqua Farms, Dade City, FL, USA).
[5] Soluble fish protein hydrolysate (Sopropeche S.A., Boulogne Sur Mer, France).
[6] Cod liver oil (MP Biomedicals, Solon, OH, USA).
[7] Refined soy lecithin (MP Biomedicals, Solon, OH, USA).
[8] Bernhart-Tomarelli mineral mix with 5ppm selenium in a form of sodium selenite (Dyets, Bethlehem, PA, USA).
[9] Custom Vitamin Mixture (mg/kg diet) Thiamin HCl, 4.56; Riboflavin, 4.80; Pyridoxine HCl, 6.86; Niacin, 10.90; D-Calcium Pantothenate, 50.56; Folic Acid, 1.26; D-Biotin, 0.16; Vitamin B12 (0.1%), 20.00; Vitamin A Palmitate (500,000 IU/g), 9.66; Vitamin D3 (400,000 IU/g), 8.26; Vitamin E Acetate (500 IU/g), 132.00; Menadione Sodium Bisulfite, 2.36; Inositol, 500 (Dyets, Bethlehem, PA, USA).
[10] L-Ascorbyl-2-Polyphosphate (Argent Aquaculture, Redmond, WA, USA).

Table 4.2. Growth performance of largemouth bass that went through different feeding regimes. The groups are as follows: 1) Saponin, programmed with saponin-supplemented feed; 2) NP-PP, programmed with SBM feed; 3) NP-FM, non-programmed group. Different letters denote a statistical difference between treatments at $P<0.05$.

Treatment	Average weight (g)	Weight gain (%)	Weight gain (g)
+ control	38.69 (±2.90)	310.13 (±41.11)[a]	29.20 (±1.72)[a]
Saponin	33.21 (±1.51)	232.55 (±41.38)[b]	23.08 (±0.50)[b]
NP-PP	35.62 (±4.53)	266.97 (±16.13)[ab]	25.87 (±2.88)[ab]
NP-FM	33.60(±0.96)	253.13 (±19.30)[ab]	24.07 (±0.92)[b]

* The (-) control was removed from the experimental trial due to high mortality and severely impaired growth caused by continuous PP diet feeding.

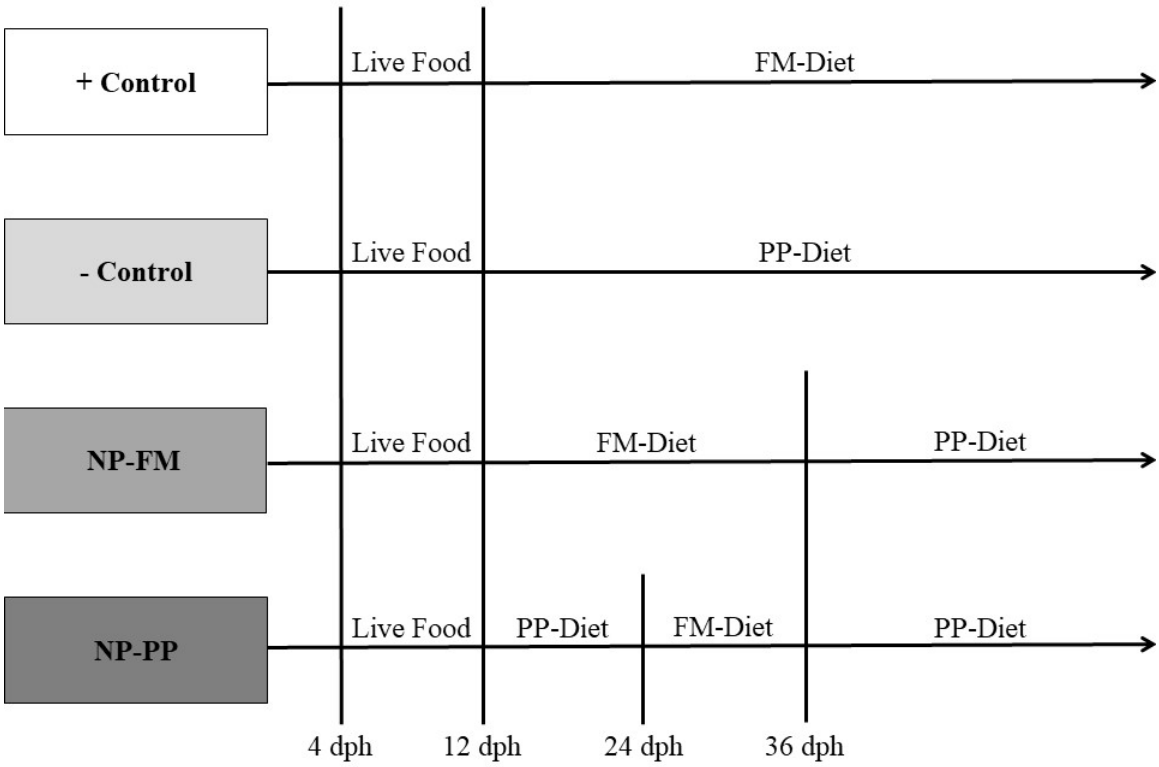
Table 4.3. The temporal differences of the gut microbiome RA (%) for the NP-PP (soy programmed group) (A) and NP-FM (non-programmed group) (B) groups of the seven most abundant Phyla of bacteria. Different letters correlate with statistical significance ($P<0.05$).

A.

Phyla	No Food (5 dph)	Post-Programming (26 dph)	Pre-PP-Challenge (84 dph)	Final (136 dph)
Acidobacteria	6.23 ±10.79	0	0	0
Actinobacteria	0	1.9 ±3.29	0	3.78±5.78
Bacteroidetes	12.89±0.55	17.86±30.93	0	0
Firmicutes	1.14±0.98	0	0	0.76±1.02
Fusobacteria	0.27±0.06	11±4.81	50.07±40.94	51.05±33.71
Proteobacteria	79.46±11.11^{ab}	71.27±29.35^{ab}	2.61±2.28^{b}	36.99±35.59^{ab}
Tenericutes	0.04±0.06	5.97±10.33	47.33±42.54	7.41±7.10

B.

Phyla	No Food (5 dph)	Post-Programming (26 dph)	Pre-PP-Challenge (84 dph)	Final (136 dph)
Acidobacteria	6.23±10.79	0	0	0
Actinobacteria	0	0	0.11±0.09	29.52±42.22
Bacteroidetes	12.89±0.55^{a}	1.67±1.67^{b}	0^{b}	0.23±.11^{b}
Firmicutes	1.14±0.98	0.14±0.25	0.18±0.17	5.18±8.97
Fusobacteria	0.27±0.06^{a}	0.17±0.07^{a}	87.25±7.49^{b}	40.02±50.54^{ab}
Proteobacteria	79.46±11.11^{a}	62.55±53.96^{ab}	3.74±2.14^{b}	7.15±5.18^{ab}
Tenericutes	0.04±0.06	35.42±55.54	9.86±6.73	17.90±29.34



FIGURES

Figure 2.1. A detailed schematic of the experimental trial in dph (days post hatch).
The four boxes represent the four treatments: (+) control which received FM (fishmeal-based diet the entire trial, (-) control which received PP (plant protein-based diet) the entire trial, the NP-FM group, or non-programmed group which received FM and then was challenged with PP at 36 dph, and the NP-PP or programmed group, which was programmed with PP, then given FM, and then challenged with PP. All groups were on trial until 65 dph.

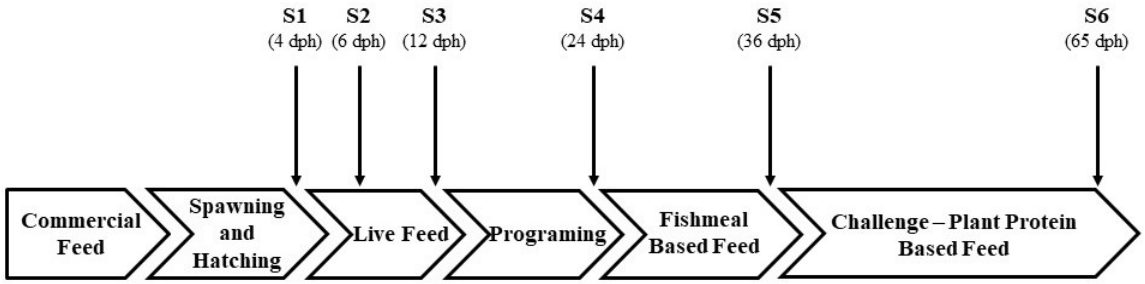


Figure 2.2. Fish sampling schedule displaying the days (days post-hatch, dph) fish were sampled. Samples of larval, juvenile, and adult fish were taken from every tank and frozen to liquid nitrogen.

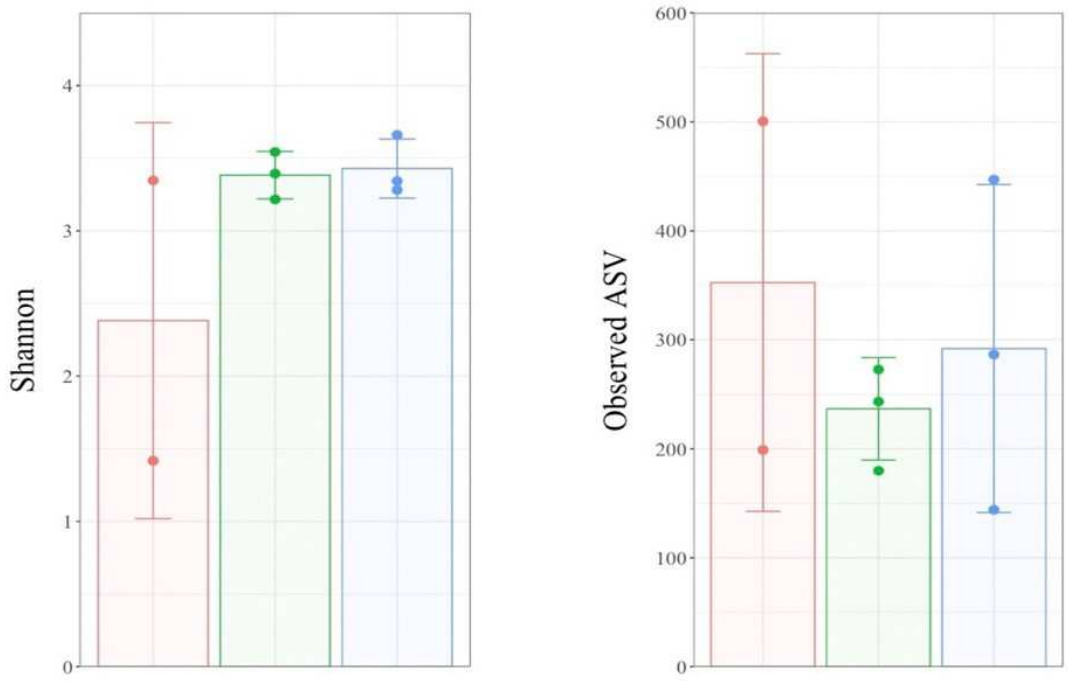


Figure 2.3. Shannon index and Observed amplicon sequence variant (ASV's) for no food (4 dph-red), rotifers (6 dph-green), and rotifers and Artemia (12 dph-blue). There was no statistical difference detected ($p>0.05$).

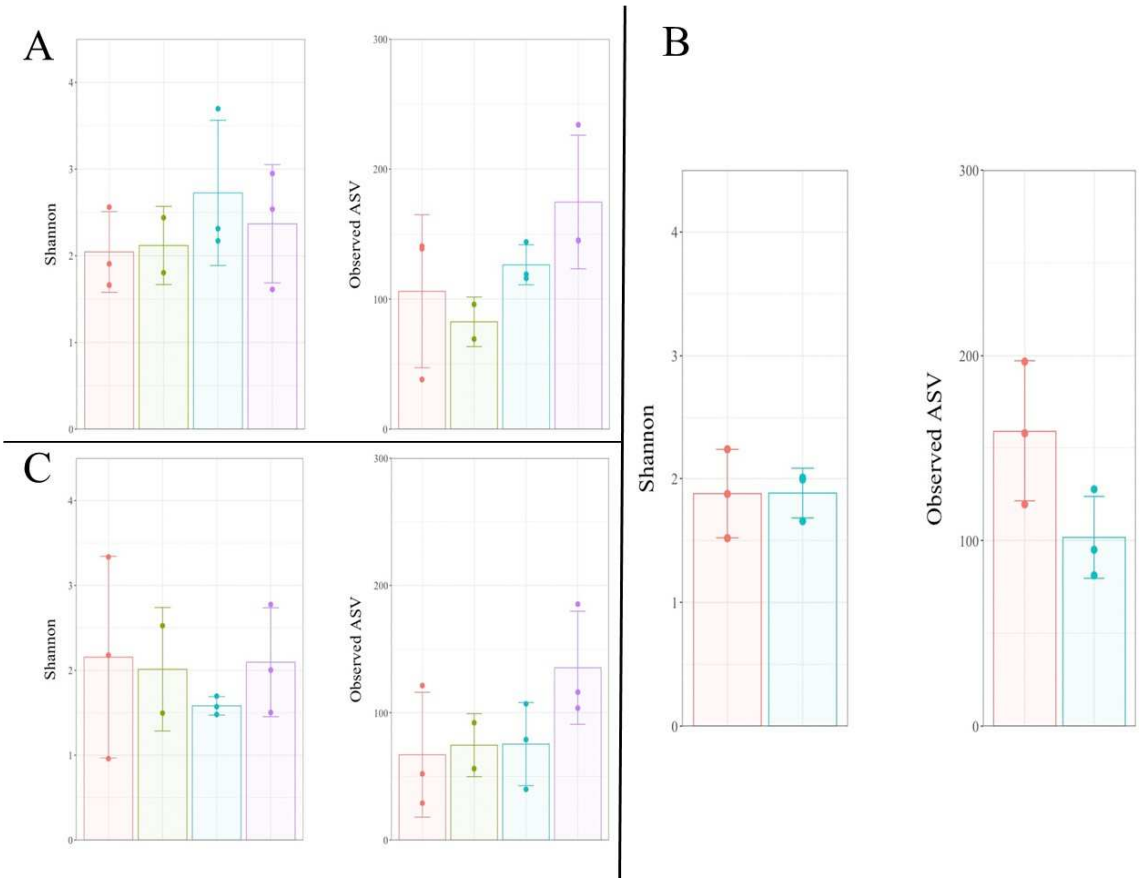


Figure 2.4. Shannon index and amplicon sequence variant (ASV's) at pre-programmed stage (24 dph, graph A), pre-PP challenge stage (45 dph, graph B) and at the final weigh point (65 dph, graph C). There was no statistical difference between the four dietary treatments detected ($p>0.05$). The red stands for the (+) control, the green is the NP-FM group, the blue is the NP-PP group, and the purple stands for the (-) control.

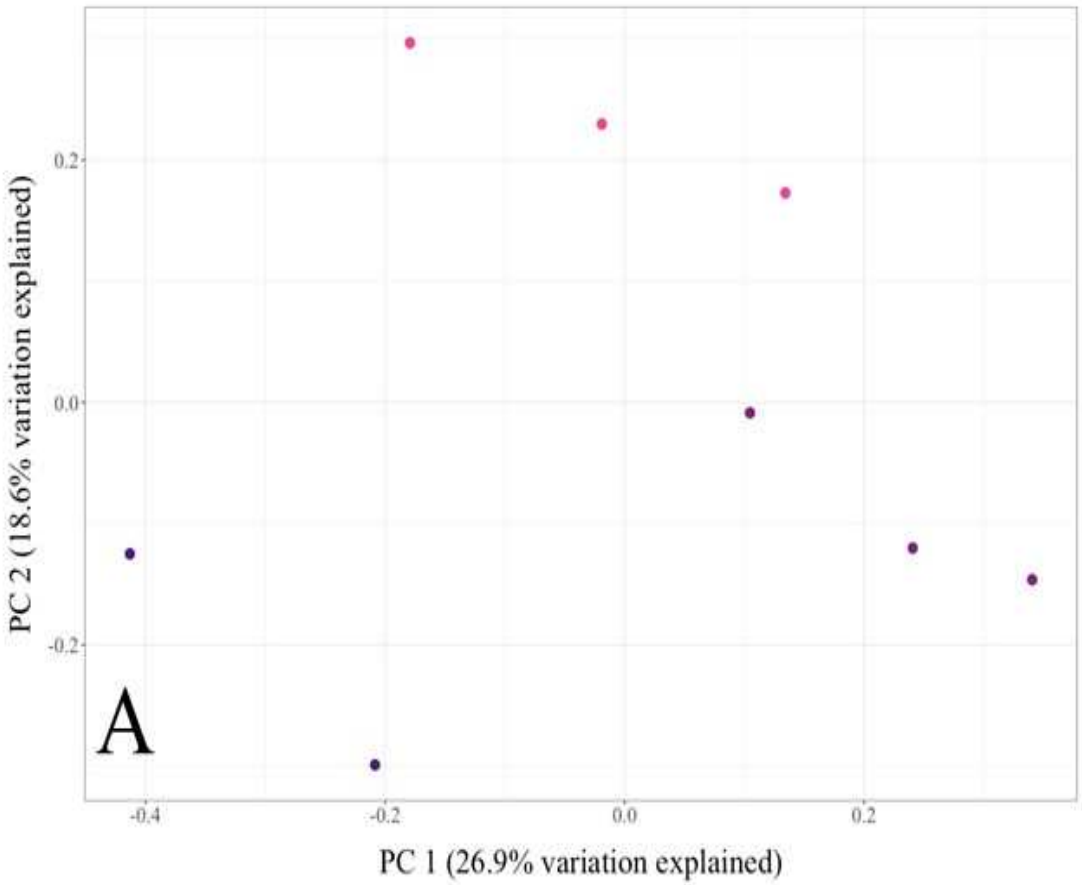


Figure 2.5. Principal coordinates analysis (PCoA) of unweighted Unifrac distances relating the variation in microbial community composition. Dark purple is 4 dph, light purple is 6 dph, and pink is 12 dph.

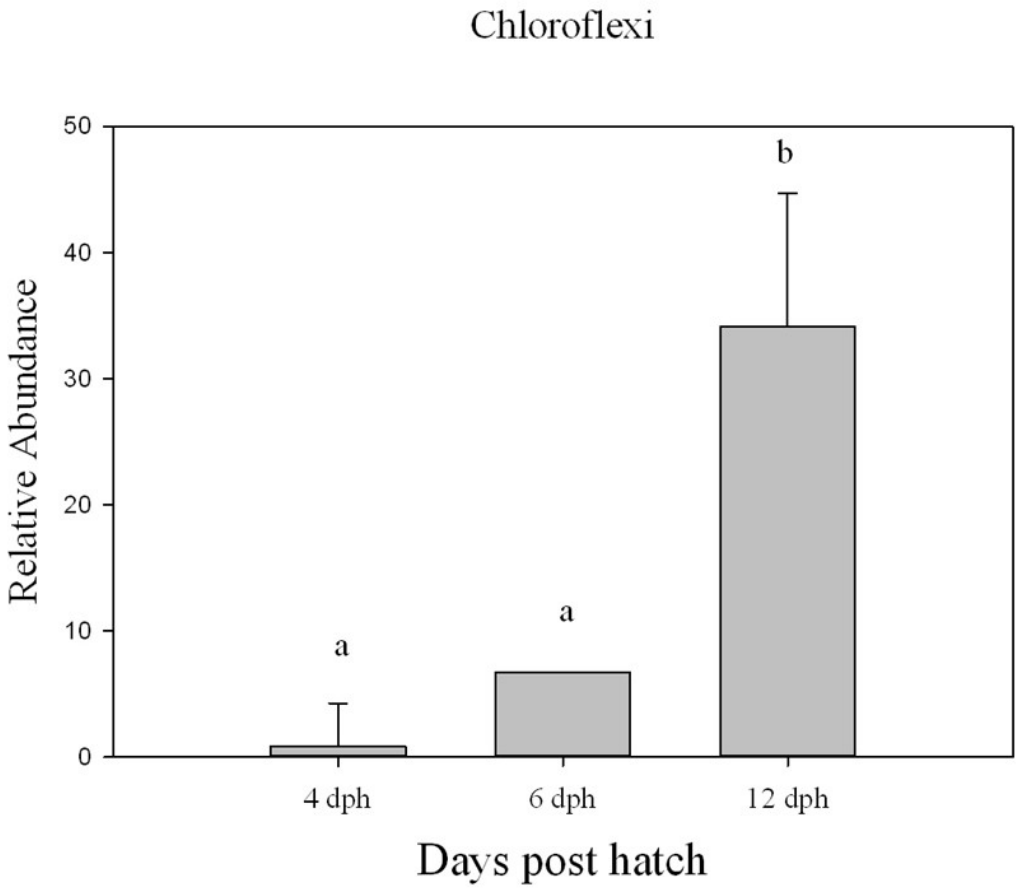


Figure 2.6. Relative abundance (%) of Chloroflexi at 4, 6 and 12 dph. Different letters indicate statistical difference ($p<0.05$). Bars are a mean of samples.

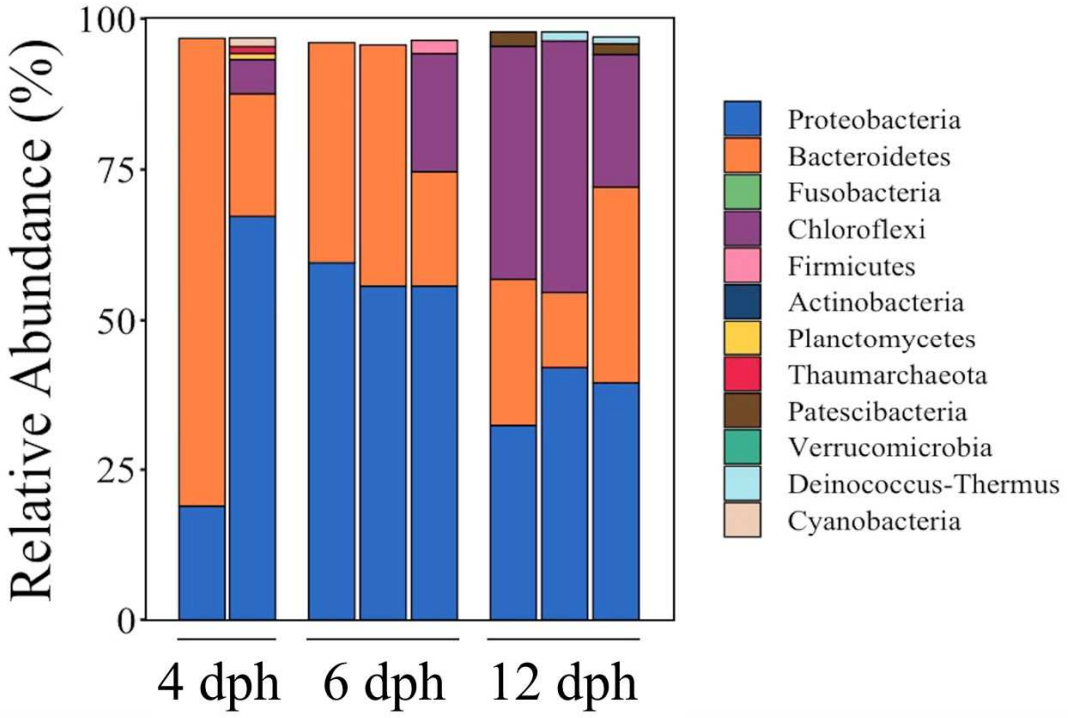


Figure 2.7. Relative abundance at 4, 6 and 12 dph. There was no statistical difference detected between groups ($p>0.05$). Different bars indicate individual samples.

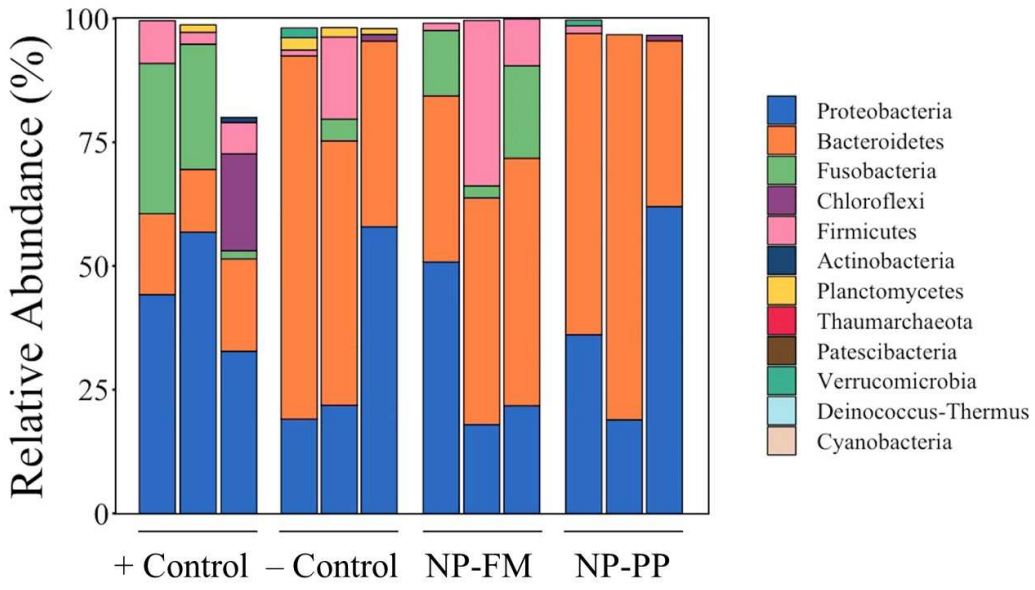


Figure 2.8. Relative abundance at 24 dph. There was a difference in RA of Bacteroidetes during the Post-programmed at this time point (24 dph). The (-) control fish had the highest amount of Bacteroidetes (54% RA), compared to positive control at 24 dph (15% RA, refer to figure 9A). Different bars indicate individual samples.

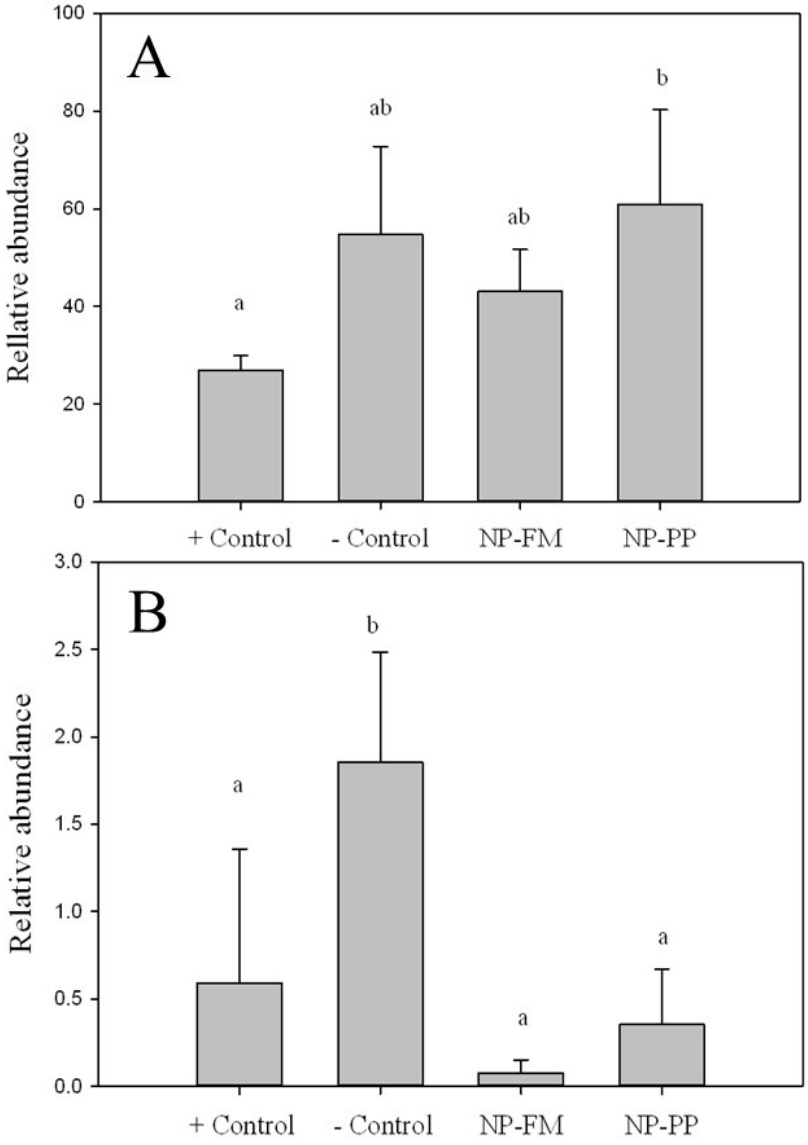


Figure 2.9. Relative abundance (%) of Bacteroidetes (A) and Planctomycetes (B) at 24 dph. Different letters indicate statistical difference ($p<0.05$). Bars are sample means for individual treatments.

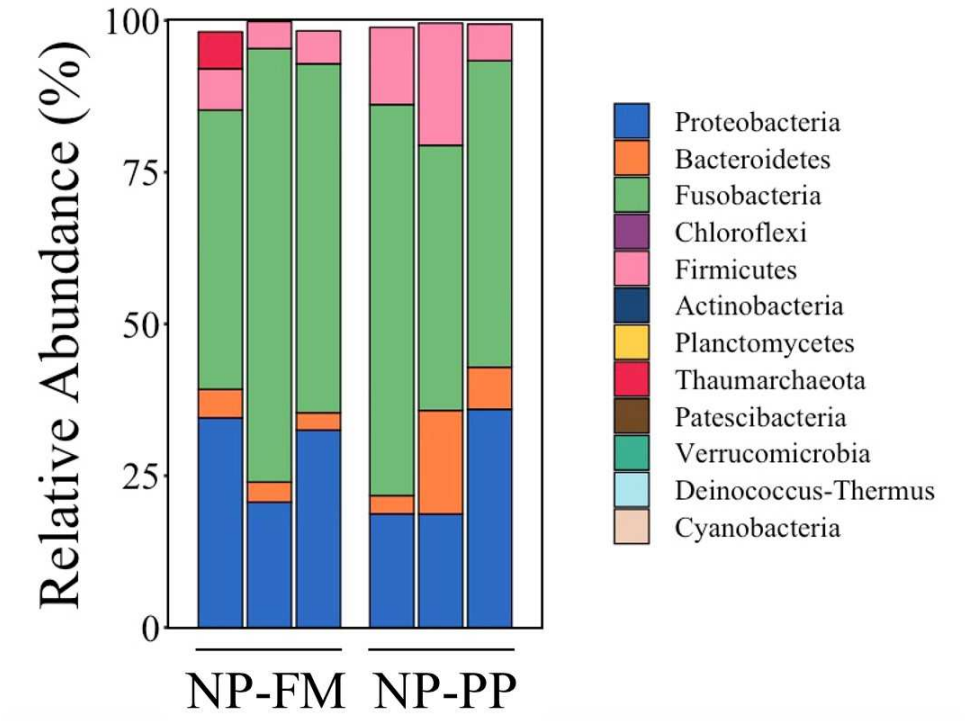


Figure 2.10. Relative abundance at 36 dph. There was no statistical difference detected (P>0.05). Different bars indicate individual samples.

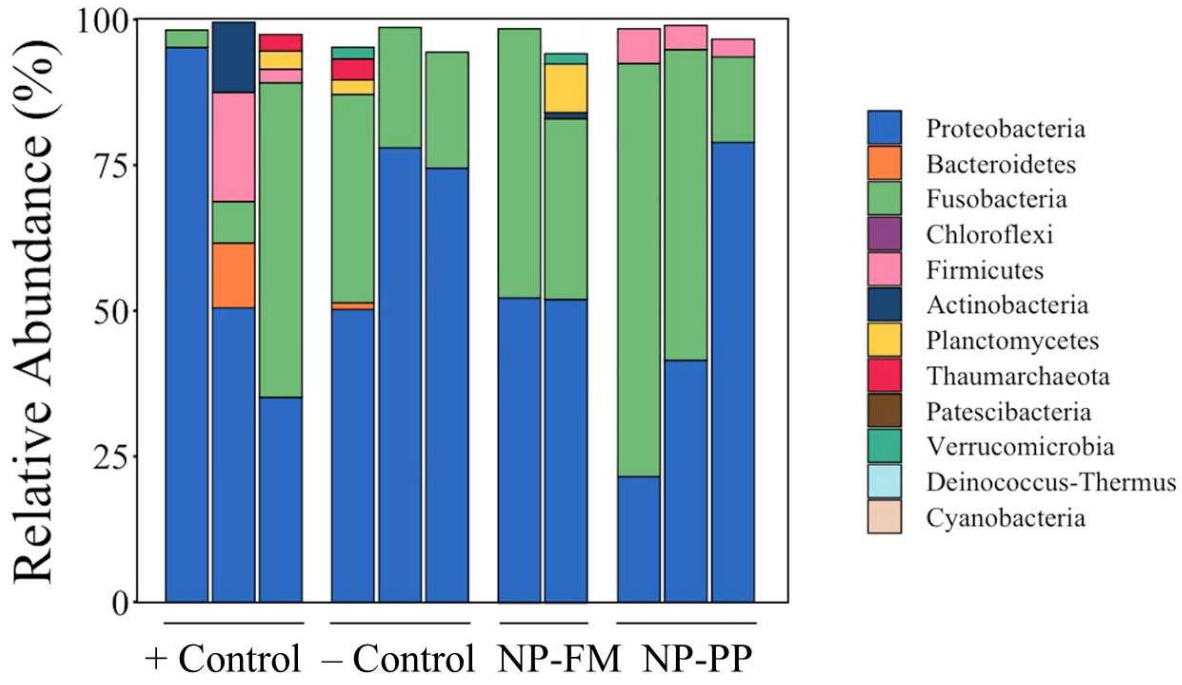


Figure 2.11. Relative abundance at 65 dph. There was no statistical difference detected (P>0.05). Different bars indicate individual samples.

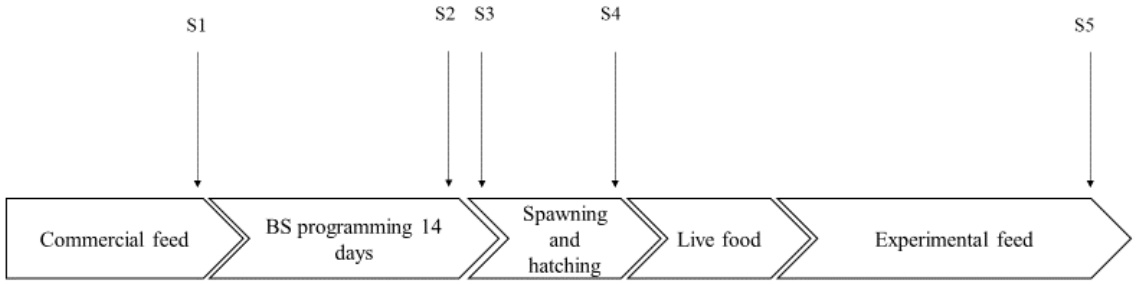


Figure 3.1. Detailed schematic presenting sampling times for gut microbiome structure.
S1 is a sampling of broodstock (BS) before programming, S2 is sampling of broodstock post two-week-programming, S3 is sampling of the eggs, S4 is sampling of larvae at mouth opening (3 days-post hatch; dph), S5 is sampling at the last day of the feeding trial (48 dph).

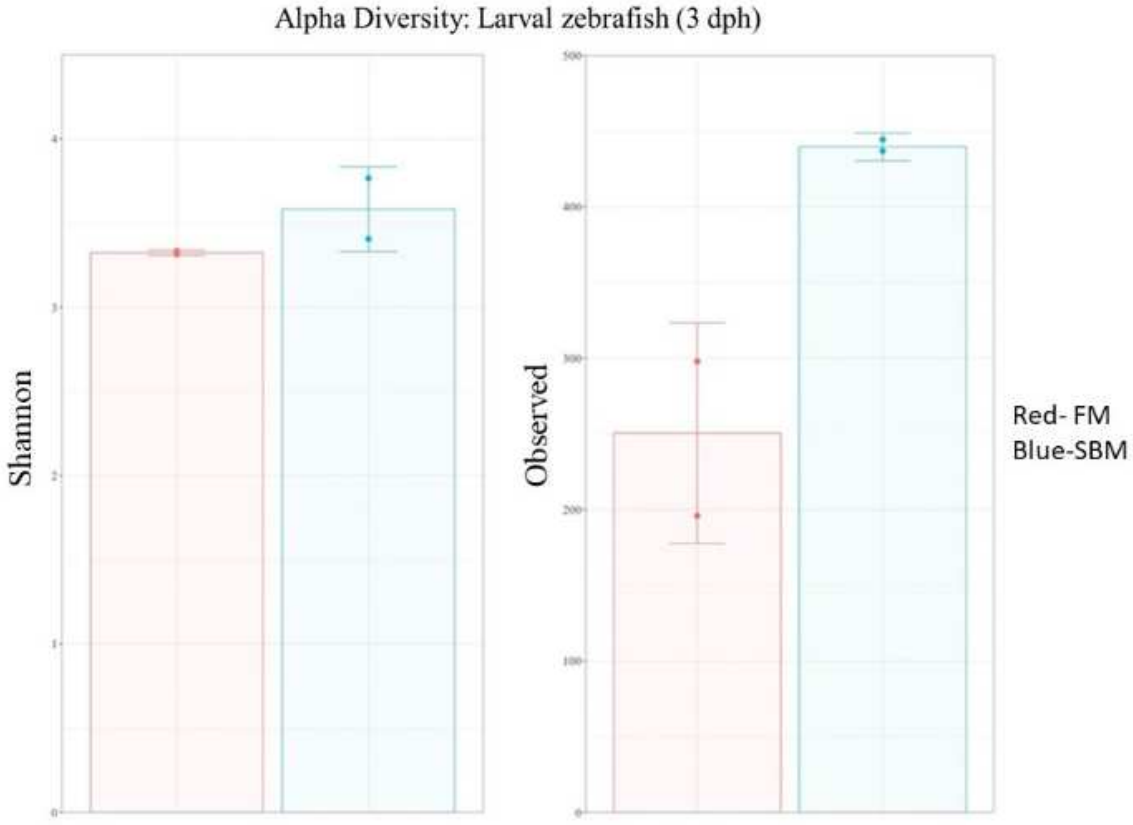


Figure 3.2. Bar plots showing the mean Shannon index value and Observed ASVs for each group at the larval (mouth opening, before the first feeding) stage with individual samples overlaid as data points. There was no statistical difference detected. Dph - days post hatch. Progeny originating from fishmeal (FM) and soybean meal (SBM) groups.

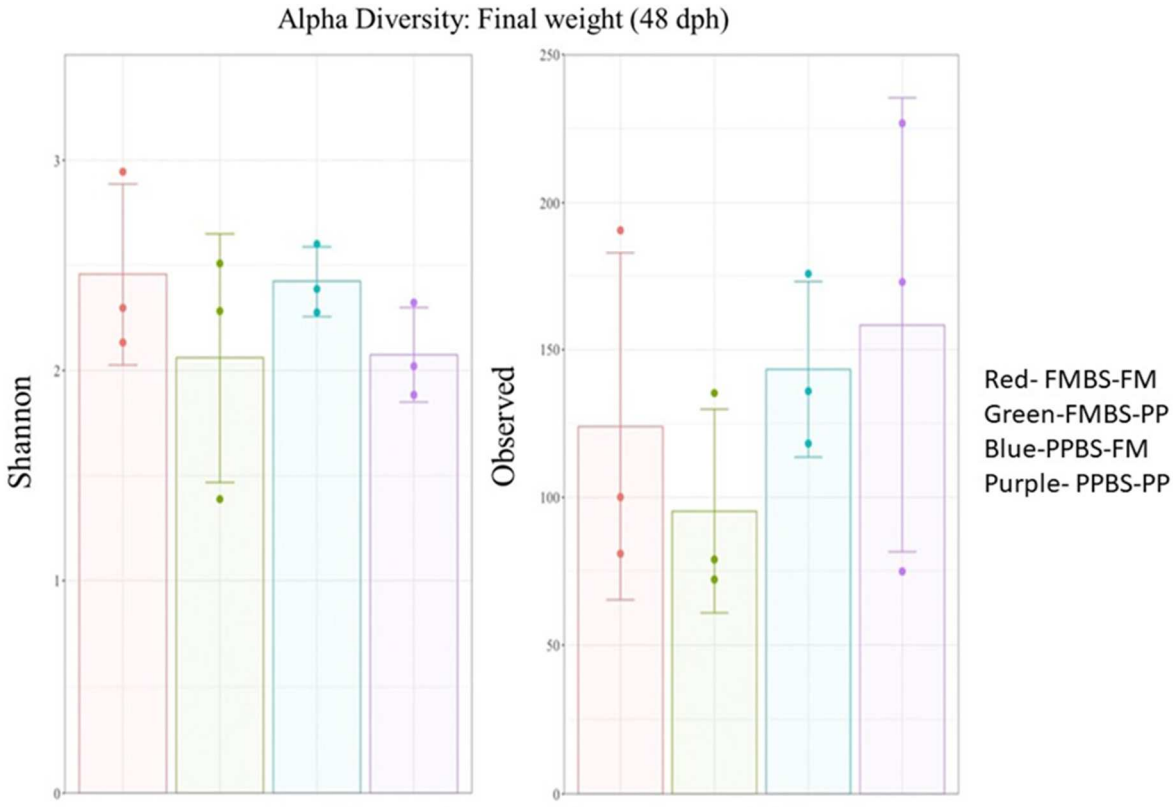


Figure 3.3. Bar plots showing the mean Shannon index value and Observed ASVs for each treatment group at the end of the study with individual samples overlaid as data points. There was no statistical difference detected. Dph - days post hatch.

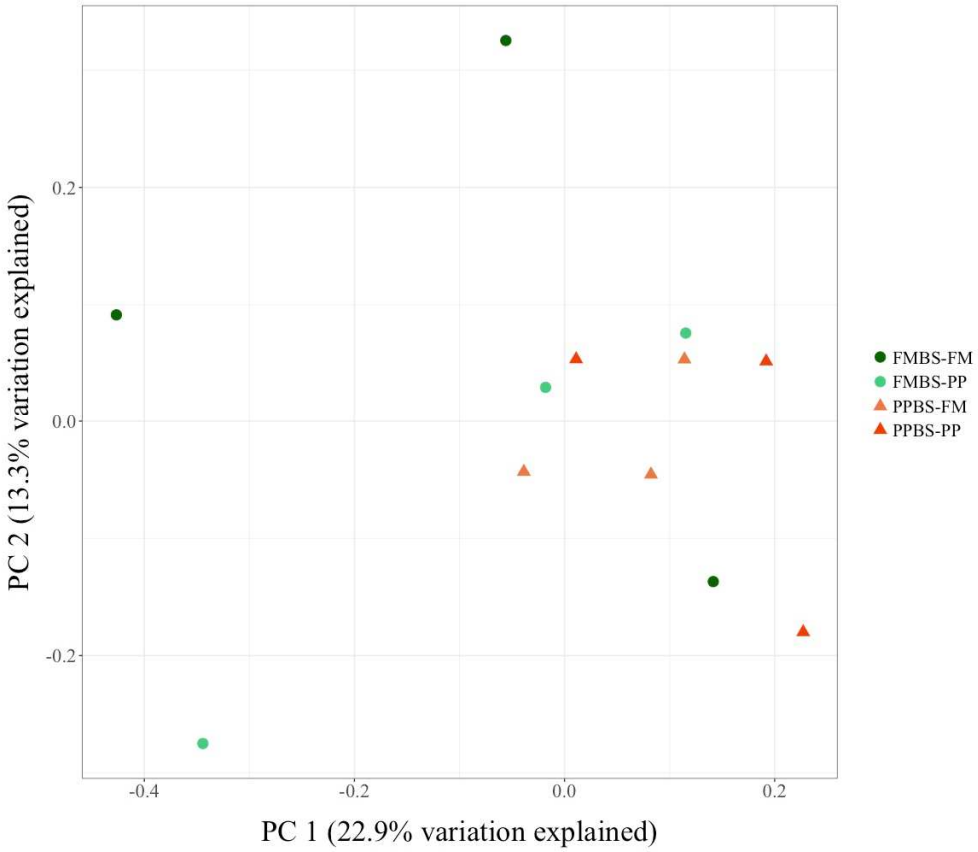


Figure 3.4. Principal coordinates analysis (PCoA) of unweighted Unifrac distances relating the variation in microbial community composition between samples from the final weights time point (48 days post hatch). Each color represents a sample group. Circles represent progeny fish samples stemming from FM broodstock, while triangles represent those from SBM broodstock. There was no significant difference detected in community composition between groups ($p>0.05$).

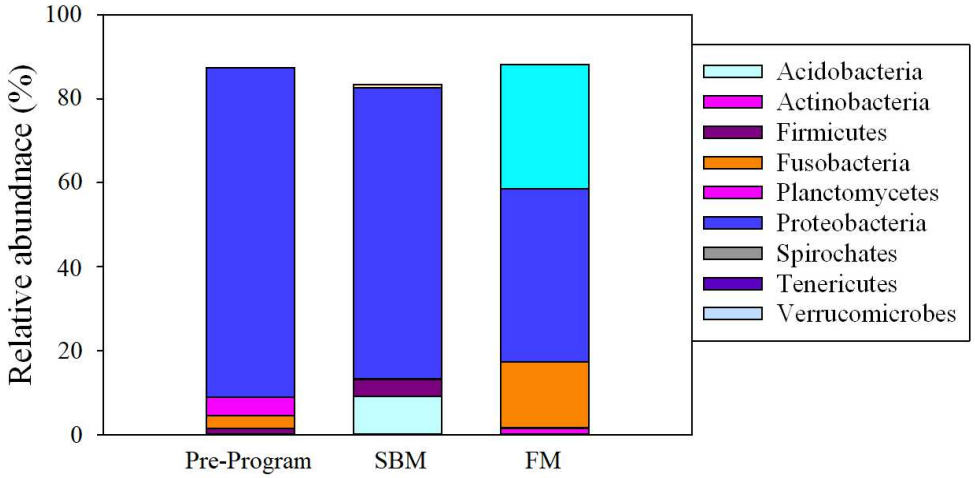


Figure 3.5. Bar plots showing the relative abundance of microbial phyla within the broodstock during the pre-program time and after the two weeks of programming with either FM or SBM diets. Abundances were normalized to the total number of sequences. Bars are a mean of samples taken n=3).

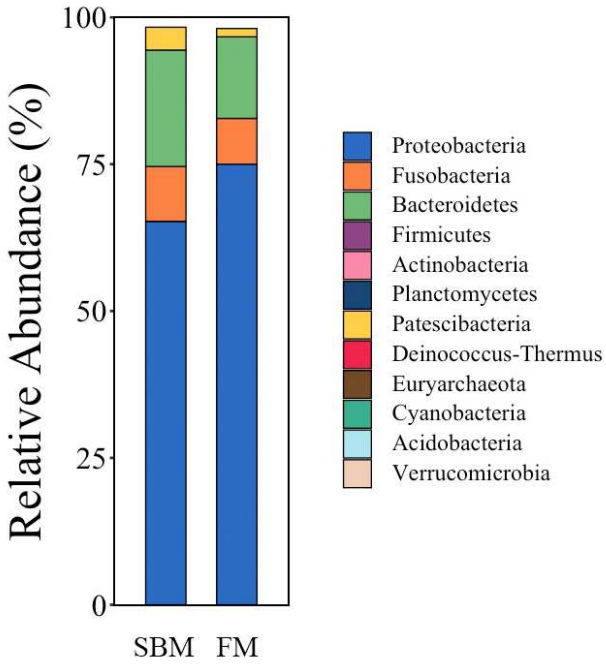


Figure 3.6. Bar plots showing the relative abundance of microbial phyla within egg samples obtained from spawning SBM-fed or FM-fed broodstock. Abundances were normalized to the total number of sequences, and phyla that constituted less than 1.0% of the overall community were omitted. Every phylum present across all samples are displayed in the legend. No significant differences were detected ($p>0.05$). Different bars indicate individual samples.

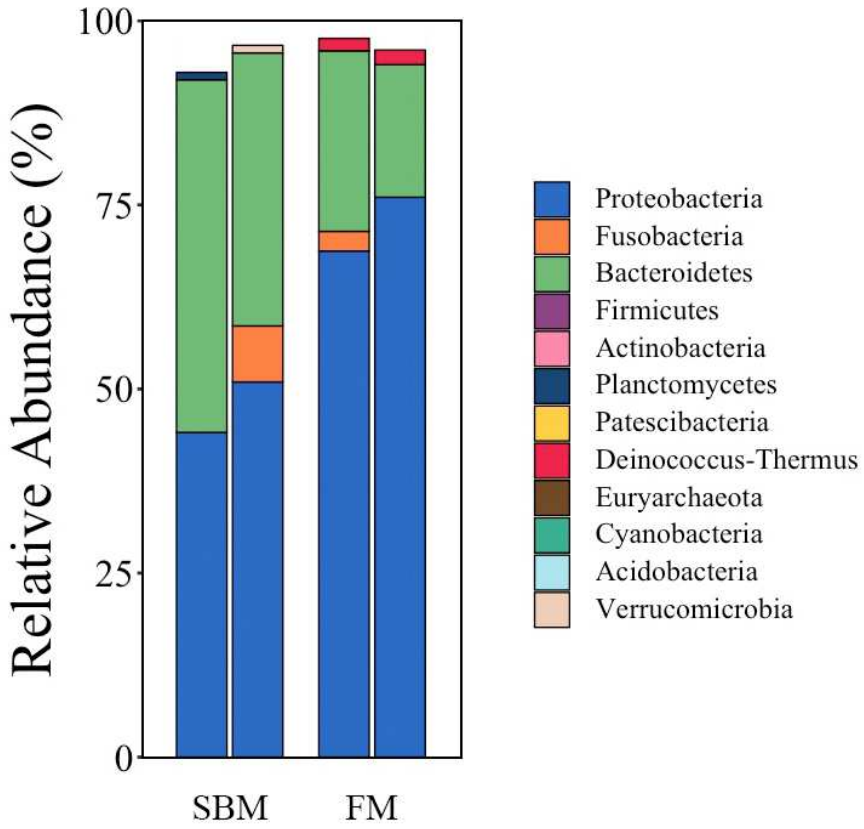


Figure 3.7. Bar plots showing the relative abundance of microbial phyla from larval samples (3 dph) that originated from spawning SBM-fed or FM-fed broodstock. Abundances were normalized to the total number of sequences, and phyla that constituted less than 1.0% of the overall community were omitted. Every phylum present across all samples are displayed in the legend. No significant differences were detected ($p>0.05$). Different bars indicate individual samples.

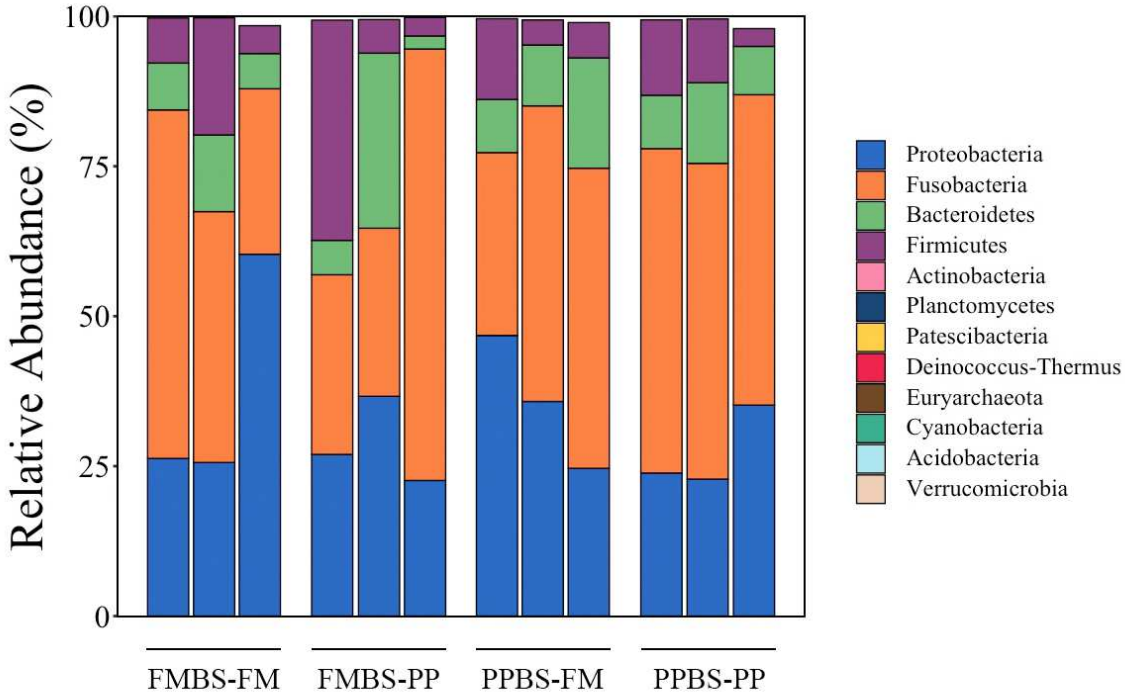


Figure 3.8. Bar plots showing the relative abundance of microbial phyla within fish gut samples from the final weights time point. Abundances were normalized to the total number of sequences, and phyla that constituted less than 1.0% of the overall community were omitted. Every phylum present across all samples are displayed in the legend. No significant differences were detected ($p>0.05$). Different bars indicate individual samples.

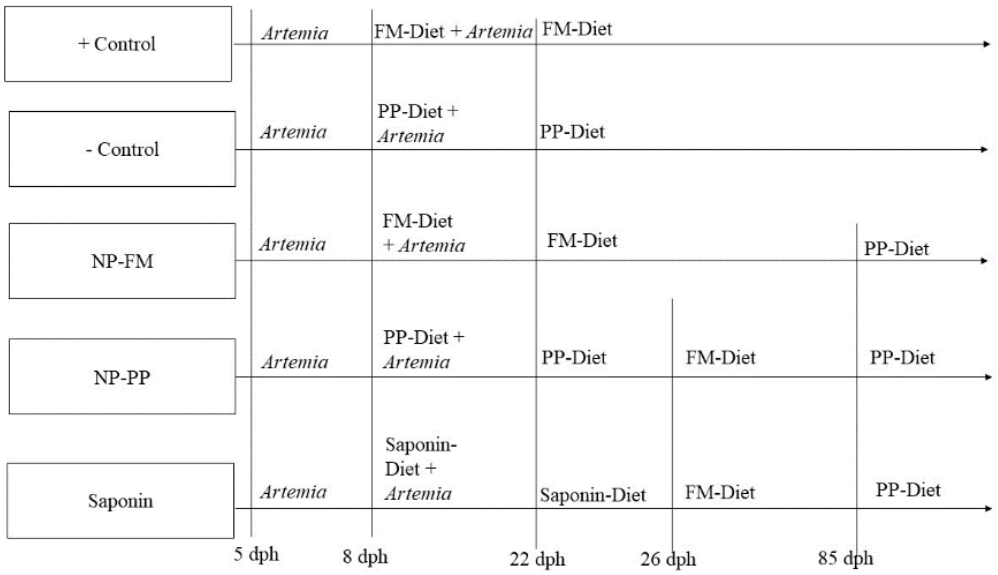


Figure 4.1. The schematic is depicted in days post hatch (dph). Fish were fed with one of three diets during the trial, a fishmeal diet (FM), a plant protein diet (PP) or a saponin-supplemented diet (Saponin).

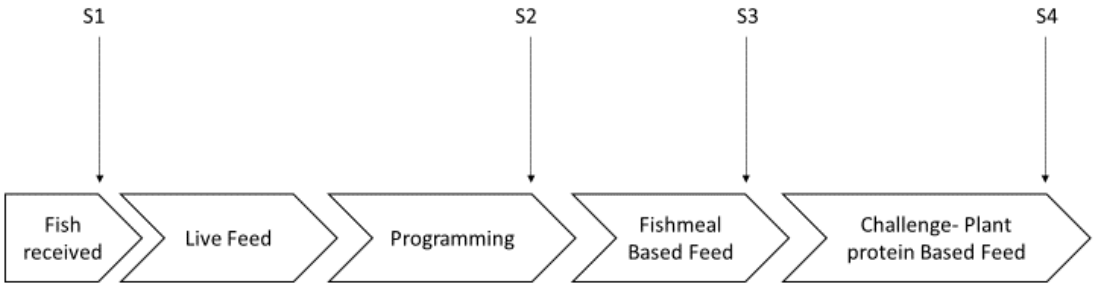


Figure 4.2. A detailed schematic showing the sampling schedule of largemouth bass gut microbiome. S1 is the sampling when fish first arrived at 5 dph, S2 is the sampling at 26 dph after programming, S3 is the sampling at 84 dph right before the PP-challenge, and S4 is the last sampling at 136 dph on the last day of the study.

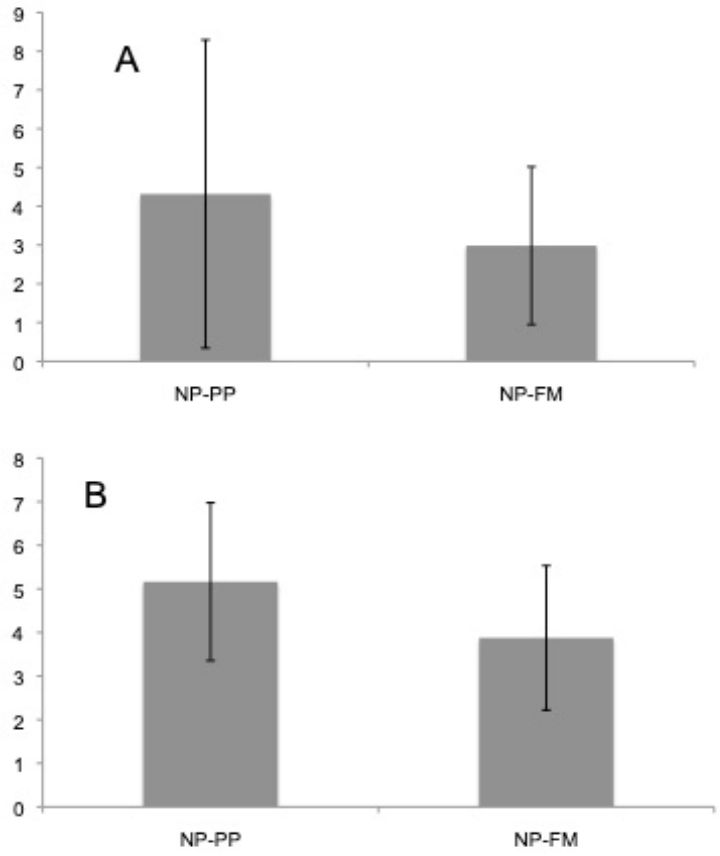


Figure 4.3. Faith Phylogenic Diversity (Faith PD) of OTUs of larval largemouth bass at post-program stage right before the PP-challenge at 84 dph (B), and at the end of the study at 136 dph (C). Faith PD is plotted against treatment, and a Kruskal-Wallis test was run to determine significance. There was no statistical difference detected between groups at any study point ($P>0.05$, $n=3$)

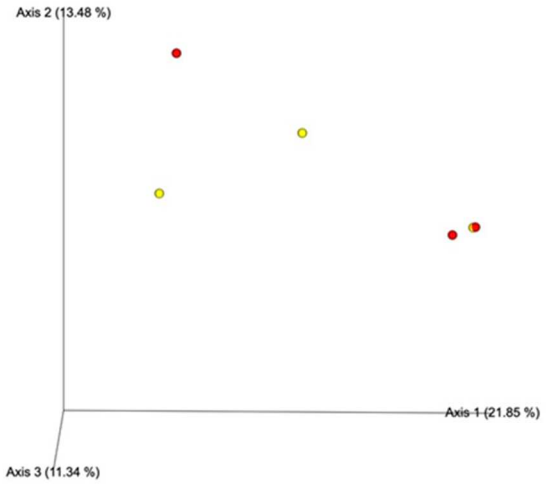


Figure 4.4. PCoA plots for the pre-PP-challenge (unweighted Unifrac) (84 dph). Yellow correlates with the NP-PP group and the red correlates with the NP-FM group. There was no significant difference detected (P>0.05).

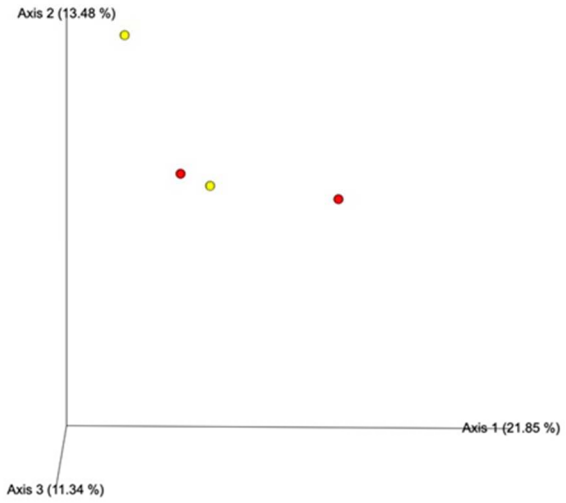


Figure 4.5. PCoA plots for samples from the end of the study (unweighted unifrac) (136 dph). Yellow correlates with the NP-PP group and the red correlates with the NP-FM group. There was no significant difference detected (P>0.05).

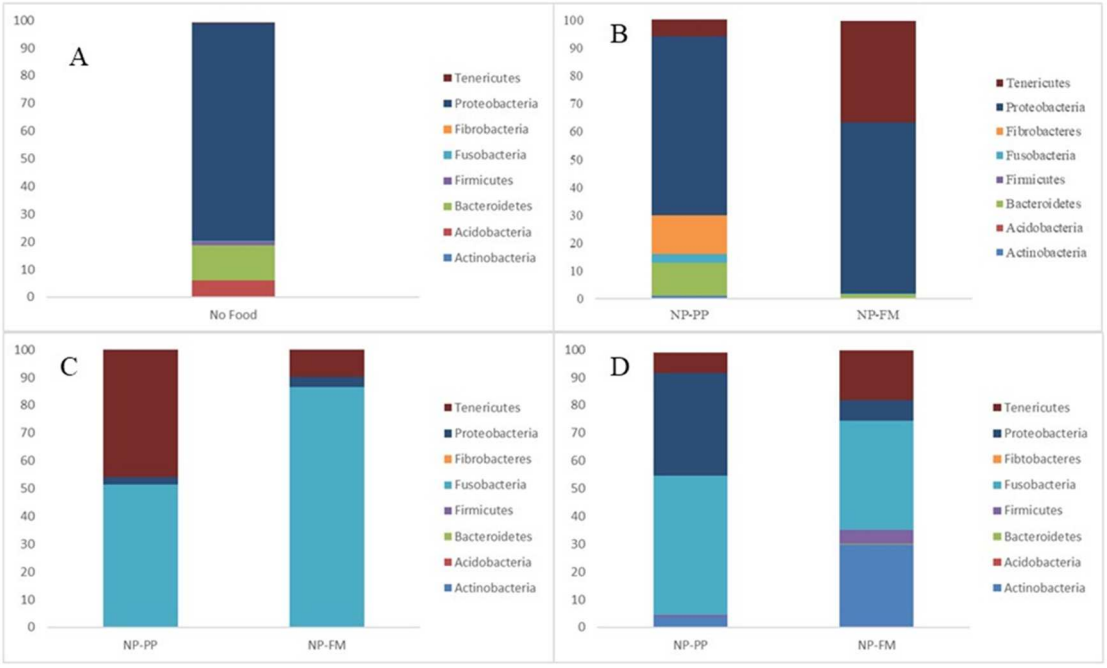


Figure 4.6. Relative abundance of the gut microbiome (%) of largemouth bass at the no food stage (5 dph, A), after programming (26 dph, B), right before the PP-challenge (84 dph; C), and at the final study point (136 dph, D). The bars for each group represent the mean of the samples taken.

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